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CYTOCHEMICAL CHARACTERIZATION OF THE GRANULES IN ZONA GLOMERULOSA OF THE BOVINE ADRENAL GLAND[1]

RICHARD L. SCHULTZ[2] AND ROLAND K. MEYER
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Madison, Wisconsin*

Spheroidal acidophilic cytoplasmic granules in the bovine adrenal gland have been described by Weber et al. ('50). These granules were confined to the zona glomerulosa of the adrenal cortex with an occasional group in the zona fasciculata. The granules were observed in the adrenal of animals as young as three weeks of age and were present in both male and female cattle. The female adrenal gland showed no noticeable change in granule content and distribution during the complete estrous cycle or in pregnancy. These were called "azocarmine granules" in the bovine adrenal by Nicander ('52) who stated that a few "azocarmine granules" were present in the glomerulosa zones of sheep, pig and female rat adrenal glands.

Fuchsinophilic granules have been observed in the adrenal in cases of adrenal virilism in cattle (Garm, '49). These granules were not found in endocrinologically normal cows. The histological structures of the zona glomerulosa of domesticated ungulates were described by Elias ('48) but cytoplasmic granules were not mentioned.

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[1] This investigation was supported in part by a research grant G-371(C6) from the National Institutes of Health, and by grants from Sharp and Dohme Inc. and the Wisconsin Alumni Research Foundation.

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In other forms, spherical or irregular fuchsinophilic bodies were found in the zona glomerulosa of sheep (Nahm and McKenzie, '36). The number of such bodies varied with the stage in estrus. A small number of such bodies were also present in the other zones of the cortex. Fuchsinophilic granules in malignant cortical suprarenal tumors of the human female have been described (Sudds, '40). Fuchsinophilic granules were found in the adrenal cortex in cases of clinical virilism of the human female (Broster et al., '38; Mellgren, '45). No other forms have been described in which the normal adrenal glomerulosa possesses cytoplasmic granules similar in distribution and other characteristics to those present in cattle.

The data to be presented in this report have been obtained from a study designed to characterize the cytological and enzymological nature of the granules in the zona glomerulosa of the bovine adrenal.

A. Histological observations

MATERIALS AND METHODS

All the adrenal tissues studied were obtained from a local abattoir and were removed from the animal approximately one half hour after killing. The adrenals were either subjected immediately to fixation or were chilled for fresh tissue studies.

For general observation, sections of the adrenal were fixed in mercury-formal-saline fluid as described by Dawson and Friedgood ('38), embedded in paraffin, and sectioned at 6 μ. The majority of the tissues was stained with Crossman's modification of Mallory's triple stain. A few were stained with Harris' hematoxylin and eosin.

Observations were made on triple stained section of adrenal glands from male and female cattle to determine whether there is any difference in giant granule content which can be correlated with the sex of the animals. Sections of glands from 14 heifers, 14 adult cows, 14 steers, and 17 bulls were studied.

The glands were classified arbitrarily according to the number of granules observed.

A second group of adrenal tissue was fixed in Regaud's and Helly's fluids, embedded in paraffin, and sectioned at 4 μ. These sections were stained for mitochondria with the anilin acid fuchsin method as outlined in Guyer ('36).

Another series of cortical tissues were fixed in Carnoy's fluid, embedded in paraffin, and sectioned at 5 μ. These sections were stained for protein with brom-phenol blue according to Mazia et al. ('53).

The fourth fixative used was 10% formalin. These sections were embedded in gelatin and frozen sections were made at 15 μ. The sections were stained with Sudan black B.

A procedure was developed to obtain a fraction of giant granules by differential centrifugation. Smears of this fraction were made and stained in order to determine the purity of the fraction and the identity of the granules within the fraction. The fraction smears were stained with anilin acid fuchsin method for mitochondria, Sudan black B, and Crossman's modification of Mallory's triple stain. The Feulgen stain was used to check for the presence of any contaminating nuclear material.

Thin slices of the outer regions of the cortex from fresh glands were teased in 0.88 M sucrose and stained with Janus green. Observations of the isolated cells were made immediately after the stain was added.

RESULTS AND DISCUSSION

The cytoplasmic granules were not seen with the Harris' hematoxylin and eosin stain as the granules stained the same color as the cytoplasm. With the triple stain, the granules stained with the orange G while the cytoplasm stained red. These granules ranged in size from 0.5 to 5 μ and were found almost exclusively in the zona glomerulosa with a few scattered granules in the zona fasciculata. These were termed "giant granules" to separate them in terminology from the "large granules" or mitochondria. The results from the comparison of giant granule content between male and female cattle, found in table 1, indicate that there is no observable difference in the distribution of the granules in the zona glomerulosa according to sex.

After Regaud's fixation, the giant granules stained with the anilin acid fuchsin but, probably because of incomplete fixation, no mitochondria were visible. With Helly's fixation, the giant granules also stained with anilin acid fuchsin. Small granules present in the same cells were about the same size as mitochondria and were for the most part spherical. Weber et al. ('56) reported the presence of giant granules and mitochondria within the same cells of the glomerulosa.

TABLE 1

Giant granule content of bovine adrenal glands according to sex

	–	+	++	+++	++++
Cows	2	5	5	2	0
Heifers	0	4	7	2	1
Bulls	1	5	6	3	2
Steers	1	6	5	2	0

—, no observable granules.
+, approximately 1–30% of the cells of the zona glomerulosa containing granules.
++, approximately 30–60% of the glomerulosa cells containing granules.
+++, approximately 60–90% of the cells of the zona glomerulosa with granules.
++++, over 90% of the cells of the glomerulosa with granules.

In the sections stained with brom-phenol blue for protein, the giant granules stained a dark blue against a light blue cytoplasmic background. With the Sudan black B stain, the granules showed an outer ring of black which indicates the presence of an external lipid layer.

In smears of the giant granule fraction, the giant granules stained red with anilin acid fuchsin, black with Sudan black B, and orange with Crossman's modification of Mallory's triple stain. The granules in the fraction were largely the giant granules which corresponded in size to those seen in the stained sections. There was a small number of smaller granules. The Feulgen stain showed only an occasional strand of red staining material which was not removed on further washing of the fraction.

With the fresh tissue slices stained with Janus green, the largest of the giant granules showed an outer ring of stain while the medium sized giant granules were stained completely. The staining of the largest granules was confused by the presence of small granules which took the stain and appeared to adhere to the side of the large giant granules. These small granules corresponded in size with the smallest granules which took the anilin acid fuchsin stain.

The cytological characteristics suggested that the giant granules are a type of mitochondria. Secretory granules in the guinea pig liver ranging in size from 0.5 to 2–3 μ have been described which were assumed to be enlarged mitochondria (Claude, '36, '37–'38). It was decided that another criterion of comparison should be investigated. The level of succinoxidase was selected since it has been shown that the succinoxidase activity is localized in the mitochondrial fraction (Hogeboom et al., '46; Schneider, '46).

B. Determination of succinoxidase activity

MATERIALS AND METHODS

Bovine adrenal glands were cooled immediately upon removal from the animal. The cortical tissue from 2–4 glands each time of preparation was grossly separated from the medulla and capsule and homogenized in 0.25 M sucrose with a sharp-pointed ground-glass homogenizer developed in this laboratory. The homogenate was diluted with isotonic sucrose to obtain a homogenate which was approximately 30% with respect to the weight of the original tissue. Both the dissection and the homogenization were performed in a cold room at approximately 3°C.

An aliquot of the homogenate was removed and diluted with 0.25 M sucrose to make a 5% homogenate. Aliquots of the 5% homogenate were used to determine the enzyme activity of the whole homogenate. The remainder of the homogenate was fractionated in an International refrigerated centrifuge at 3°C.

The centrifugation procedure used was a modification of the method described by Hogeboom et al. ('48). In the initial method of centrifugation the homogenate was centrifuged for 20 minutes at 230 g to remove a large part of the nuclei, red blood cells, and cellular debris. This portion was put into the discard fraction. The first supernatant was then centrifuged for 40 minutes at 1200 g to obtain a crude giant granule fraction. The crude fraction was suspended in isotonic sucrose and centrifuged at 200 g for 10 minutes to remove any remaining nuclear fragments and red blood cells. The resultant supernatant was centrifuged for 20 minutes at 1300 g, the pellet resuspended in sucrose, and centrifuged for 20 minutes at 1300 g to reduce the mitochondrial contamination. This yielded the final giant granule fraction, the relative purity of which was checked by staining smears of the fraction as described in the previous section.

The supernatant obtained from the original crude giant granule fraction was centrifuged for 20 minutes at 6900 g to obtain the mitochondrial fraction. The resultant supernatant was centrifuged for one hour at 20,400 g to give the microsome fraction. The various washings were added to the discard fraction.

A modification of the above procedure provided the final method of centrifugation. In this modification the suspended giant granule fraction was centrifuged for one minute at 1300 g to remove any residual nuclear fragments and the mitochondrial fraction was washed.

The fractions were diluted to known volumes with 0.25 M sucrose. The succinoxidase activity of aliquots from each fraction and of the whole homogenate was determined manometrically according to the method of Schneider and Potter ('43).

Concurrently nitrogen determinations were performed on aliquots from the same fractions using a modification of Johnson's method ('41). The succinoxidase activity of each fraction was interpreted on the basis of the nitrogen content.

In the first two series of determinations, a normal substrate was used in determining the succinoxidase activity. A third series was done in which a doubled concentration of substrate was used to determine whether the substrate was a limiting factor. Each series consisted of 5 determinations with 2 to 4 flasks per fraction at each time of determination.

The method of Eilert ('53) was used in determining the total lipid content of the fractions.

The "t" test was the method used for a statistical comparison of the enzyme activity of the fractions.

TABLE 2

Succinoxidase activity of fractions obtained by initial method of centrifugation [2]

FRACTION	QO_2 (μl O_2/mg N_2/hr.)	MEAN % RECOVERY	Mg N_2/gm ORIGINAL WET WT.
Whole homogenate	563.5 ± 15.5 [1]	100	23.589 ± 0.460 [1]
Giant granules	1226.2 ± 77.2	1.5	0.178 ± 0.054
Mitochondria	1607.9 ± 88.2	36.7	3.190 ± 0.132
Microsomes	457.9 ± 51.7	3.6	1.179 ± 0.137
Supernatant	40.9 ± 4.9	1.2	5.143 ± 0.175
Discard fraction	—	36.2	—

[1] Mean ± standard deviation of the mean.

[2] The giant granule fraction was subjected to low speed centrifugation 1× and washed 2×. The mitochondrial and microsome fractions were not washed.

RESULTS AND DISCUSSION

With the initial method of preparation of the fractions, the mitochondrial fractions had the highest succinoxidase activity of all the fractions. The giant granules fraction was shown to have enzyme activity somewhat less than that of the mitochondria, but greater than that of the microsome fraction. Both differences were highly significant. The microsome fraction had greater activity than the supernatant. The results are found in table 2.

When the succinoxidase activity of fractions obtained by the final method of centrifugation was determined, again the mitochondria showed a higher enzyme activity than the giant granules. The giant granules had a greater activity than the microsomes. These differences were highly significant. The data are found in table 3.

Calculations were also made to determine the enzyme activity of the whole homogenate and each of the fractions per gram of wet weight of the cortex. The homogenate activity was taken as 100 percent and that of each fraction as percent activity of the whole homogenate. The total of the percent activities of the fractions gave the percent recovery which is also shown in the tables.

TABLE 3

Succinoxidase activity in fractions obtained by final method of centrifugation[2]

FRACTION	QO_2 (μl O_2/mg N_2/hr.)	MEAN % RECOVERY	Mg N_2/gm ORIGINAL WET WT.
Whole homogenate	570.6 ± 40.1[1]	100	24.710 ± 0.481[1]
Giant granules	1226.1 ± 112.2	1.7	0.220 ± 0.017
Mitochondria	2603.4 ± 150.3	39.6	2.373 ± 0.164
Microsomes	428.4 ± 12.9	3.7	1.292 ± 0.165
Supernatant	36.5 ± 2.4	1.3	5.485 ± 0.232
Discard fraction	—	50.2	—

[1] Mean ± standard deviation of the mean.

[2] The giant granule fraction was centrifuged at low speed 2× and washed 2×. The mitochondrial fraction was washed 1× and the microsome fraction was not.

Comparisons were made between identical fractions of the two methods of preparations. It was found that there was no significant difference between the enzyme activity of the giant granules of the two preparations, while there was a highly significant difference between the succinoxidase activity of the mitochondria. This comparison demonstrates that the additional step in the preparation of the giant granules did not yield any significant difference in enzyme activity. The mitochondrial fraction however, showed a much greater enzyme activity per mgm of nitrogen after washing. A similar increase in succinoxidase activity of liver mitochondria after washing the fraction has been observed, (Hogeboom et al., '48).

When the succinoxidase activity of the fractions in a doubled concentration of substrate was compared statistically with the activity of the fractions in a normal substrate, it was found that there was no significant difference between the mitochondrial fractions or between the giant granule fractions. The comparison shows that the substrate is not a limiting factor.

Considerable investigation has been done concerning the heterogeneity of the mitochondrial fraction. Two morphologically and biochemically distinct types of mitochondria were observed in a number of tissues including the human adrenal (Laird et al., '52). No granules comparable in size to those discussed in this paper were described. Novikoff et al. ('53) described long mitochondria from rat livers ranging in length from 1.5 to 5 μ. The fraction in which the long mitochondria were the most numerous showed the maximum succinoxidase activity. The mitochondria fraction from mice livers was divided into 4 fractions by Kuff and Schneider ('54). The main bulk of the succinoxidase activity was found in the fractions containing the larger mitochondria but the authors were unable to find any clear-cut correlation between morphology and enzyme activity. When mitochondria from rat livers were divided into 4 fractions by Paigen ('54), the heaviest fraction gave a marked succinoxidase activity but the maximum enzyme activity was shown by the lighter fractions. There is no mention as to whether this difference is statistically significant.

The total lipid showed extreme variation. There was no similarity in the proportion of lipid in the fractions between different runs. This extreme variation in the lipid content is not too surprising since the animals were subjected to a number of uncontrollable conditions, such as diet and stresses. The fat content varies greatly in the adrenal depending on the state of the animal (Hartman and Brownell, '49).

These data indicate that perhaps the giant granules present in the zona glomerulosa of cattle adrenals are mitochondria. The staining properties of the giant granules are similar to the mitochondria and the giant granules also contain a substantial amount of the succinoxidase activity which is considered to be confined to the mitochondria. The physiological significance of these granules is still undetermined. Some observations on the biological activity of the granule fractions are being reported elsewhere (Schultz and Meyer, '58).

SUMMARY

1. Giant granules are described in the cytoplasm of the cells of the zona glomerulosa in the beef adrenal gland. The granules were shown to stain with mitochondrial, lipid, and protein stains.

2. There is no apparent difference in the concentration of granules dependent on the sex of the animal.

3. A centrifugation procedure for the isolation of a giant granule fraction is described.

4. The giant granule fraction shows a positive succinoxidase activity. This activity is considerably less than that of the mitochondrial fraction, but far greater than the enzyme activity of the microsome fraction.

5. The mitochondrial nature of the giant granules is discussed.

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THYROID REGULATION OF PROTEIN AND NUCLEIC ACID ACCUMULATION IN DEVELOPING SKELETAL MUSCLE OF THE CHICK EMBRYO [1]

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Laboratory of Chemical Embryology, University of Colorado, School of Medicine

TEN FIGURES

INTRODUCTION

Hormonal regulation of development appears to manifest itself by eliciting differential responses on the part of cells in which certain differences have already been established. This is particularly evident in the action of the sex hormones (Burns, '47; Willier, '52; Jost, '53) but is not without confirmatory data from studies of other endocrine influences (Geigy, '41; Weiss and Rossetti, '51; Etkin, '55). This conclusion, has been arrived at, in fact, on purely theoretical grounds (Weiss, '39, p. 440 et seq.).

The advantages of exploring hormonal regulation lie in defining these differences among the responding cells with the ultimate aim of examining the mechanisms responsible for their establishment. Of equal importance is the opportunity offered by endocrine correlations of analyzing the manner in which initial differences are expanded in the course of development.

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[1] Supported by grants from the National Science Foundation and the U.S. Public Health Service (Grant B-549).

The fact that hormonal realization of these latent differences occurs at relatively late stages of development (Willier, '52 and '55) should not constitute an insurmountable difficulty, since the technique of "backward projection" has, in the past, proved a fruitful approach in the development of embryology.

It has been amply demonstrated that injections of goitrogenic compounds produce cytological changes in the embryonic thyroid gland (Adams and Bull, '48; Vidal, '52) similar in every respect with those produced in weanling rats and indicative of inhibited function (Mackenzie and Mackenzie, '43). In such thyroid deficient embryos the augmentation of body weight is markedly depressed (Adams and Bull, '48; Vidal, '52).

The present study was undertaken to determine the effects of the suppression of thyroid activity on the composition of skeletal muscle tissue in terms of its protein and nucleic acid content. A future extension of this approach to the problems of organogenesis and cellular differentiation is feasible since previous investigations in this laboratory have elucidated the normal accumulation rates of various muscle proteins (Csapo and Herrmann, '51: Herrmann and Barry, '55; Konigsberg and Herrmann, '55).

A decreased rate of protein accumulation becomes evident between 4 and 6 days after treatment with thiourea on the 10th day of development. Although initially this inhibition may be related to an alteration in ribonucleic acid content, the progression of this defect is evidently not explicable in terms of ribonucleic acid content alone.

A decrease in nuclear number, inferred from the accumulation pattern of deoxyribonucleic acid, also occurs, but is transitory in character and is probably not related to the goitrogenic effects of thiourea.

METHODS

Throughout the course of this investigation White Leghorn-Delaware embryos (Hy-Line Strain), incubated at 37.5°C in a forced-draft incubator, were used.

Thiourea (freshly prepared from Merck reagent-grade crystals) was administered intravenously on the 10th day of development. Each embryo received a total of 8.4 mg given as a 2% solution. The junction of the allantoic vein and the chorioallantoic membrane was visualized by transillumination. One of the major blood vessels radiating out from this point was marked and exposed by sawing a "window" over the vessel and applying paraffin oil to the intact shell membrane. A small glass needle fixed to a hypodermic syringe and needle was introduced into the blood vessel with the aid of a simple manipulator whose basic component is a vertically mounted mechanical stage. Volumes were measured and delivered by a micrometer mounted in such a fashion that its spindle was in contact with the syringe plunger. (A similar technique is described in the literature (Goldwasser and Shelesnayk, '53). Control embryos received an injection of an equimolar amount of urea or NaCl or an equal volume of distilled water.

Thyroxine was administered similarly in the reversal series. Graduated doses were given daily from the 13th through the 18th day of development. Each daily dose was proportional to the average body weight of normal embryos of the same developmental age. The various injection series are designated by the final total dose, characteristic of the series. (Total Dose $= x + xe^{2k} + \ldots + xe^{nk}$, where x is the initial dose and k the rate constant (0.240) of the growth of body weight in normal embryos.)

Crystalline thyroxine (Squibb) was made up as a 250 μg per cm^3 solution in dilute NaOH and stored in a deep freeze at —10°C. Individual vials of the solution were thawed and diluted immediately prior to use.

The embryos were sacrificed at 2-day intervals following treatment with thiourea. Body weights were measured after blotting with rough paper toweling. At the older stages care was exercised to remove all of the retracted yolk sac before weighing.

Muscle tissue was removed and homogenized under conditions described previously (Konigsberg and Herrmann, '55), except that all of the leg musculature was removed, as quantitatively as possible, from the tendinous insertions at the tarsal portion of the tibia to the insertions at the ilial and ischial crests. Total muscle tissue was weighed rapidly on a Roller-Smith balance prior to freezing.

Duplicate aliquots of 0.25 cm^3 of the homogenates were taken for determinations of protein nitrogen. Half cubic centimeter portions were used for determinations of DNA and RNA. The nucleic acids were extracted by Schneider's ('45) procedure. DNA was determined by Dische's ('30) diphenylamine method and RNA by the method of Euler and Hahn ('46). Optical densities were read at the respective absorption maxima in the Beckman model DU spectrophotometer. Standards were prepared from commercial samples of calf thymus deoxyribonucleic acid and yeast ribonucleic acid (obtained from Schwarz Laboratories, N.Y.). The fractions reserved for the determination of protein nitrogen were carried along with the DNA samples and treated identically, except for the omission of the extraction with hot TCA.

After digestion and nesslerization the optical density of the nitrogen samples was read in a Klett-Summerson colorimeter at 500 μ.

The histological sections were prepared from thyroids fixed in Bouin's fluid. All sections were cut at 5 μ except for the organs taken from 12-day embryos, which were sectioned at 2.5 μ. Routine hematoxylin-eosin staining was used. In some cases staining with Schiff's reagent after periodic acid oxidation (McManus, '48) was also employed.

The statistical treatments used are those described by Mather ('47).

RESULTS

The dosage of thiourea employed was chosen after a preliminary study in which the amount of the goitrogen injected at the 10th day was varied from 2.1 to 15.0 mg. Embryos were sacrificed on the 18th day and body weights were taken. With quantities greater than 10.8 mg the mortality was 100%.

No appreciable further decrease in body weight was observed by increasing the dose above 8.4 mg although a progressive increase in mortality did occur.

Detailed studies of the cytological effects of goitrogenic agents on the thyroid gland of the chick embryo have been reported (Adams and Bull, '49; Adams and Buss, '52). Since the injection protocol used in this study differs from those used in the investigations cited above, a histological examination of the thyroid gland seemed warranted.

The histological evidence (see plate 1) indicates that in terms of colloid depletion the effect of thiourea administration can be detected as early as the 12th day and persists at least until the 20th day. Those traces of colloid which can be observed in the thyroids from thiourea-treated embryos stained by the PAS technique do not necessarily indicate the presence of the normal gland product even in trace amounts (Dempsey, '49).

Body weight determinations agree essentially with those reported by Vidal for his doubly injected group (Vidal, '53). Figure 1 demonstrates that body weight increases more slowly in thiourea-treated embryos after the 12th day of development. At the 14th day the difference in body weight between control and thiourea-treated embryos (16%) is significant below the 1% level (table 1). The difference increases progressively to the 20th day at which time the body weight of the thiourea-treated group is 35% lower than the control value.

In contrast to the body weight no statistically significant difference can be demonstrated in muscle fresh weight until the 16th day (fig. 1), at which time a 20% difference between experimentals and controls is significant below $p = 0.01$ (table 2). Prior to this time, at the 14th day, the muscle weight of the experimental group is only 7.7% below the control level. Even disregarding the extremely low probability of this difference (0.4) these data indicate, at the very least, that any reduction in the leg musculature alone would not account for the inhibition of body weight at this time. Thus, some other organ or organs are affected by thiourea treatment before an effect on muscle fresh weight can be detected (see Romanoff and Laufer, '56).

A comparison of the DNA content of the leg musculature in experimental and control groups should indicate to what extent the difference in muscle fresh weight can be accounted for by a decrease in "cell number." (In dealing with muscle tissue the assumption must be made that despite the multinuclear nature of the cells a discrete relationship exists between each nucleus and its dependent volume of cytoplasm. Thus, as has been pointed out previously (Herrmann et al., '56), it would be more correct to speak of nucleocytoplasmic units rather than cell numbers in this tissue.) Table 3 indi-

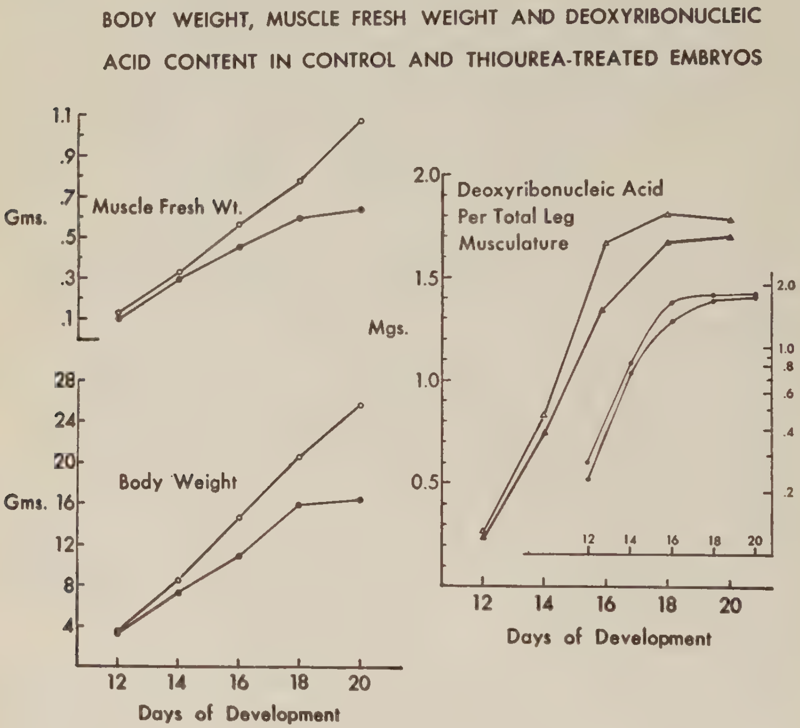
BODY WEIGHT, MUSCLE FRESH WEIGHT AND DEOXYRIBONUCLEIC ACID CONTENT IN CONTROL AND THIOUREA-TREATED EMBRYOS

Fig. 1 Open figures represent the means for control embryos; closed figures represent the means for thiourea-treated embryos.

TABLE 1

Body weight (grams)

DAYS OF INCUBATION	CONTROL (I)			THIOUREA-TREATED (II)			p (I MINUS II)
	Mean ± S. E.	n	p[1]	Mean ± S. E.	n	p[1]	
12	3.44 ± .13	6		3.16 ± .12	6		> 0.1
14	8.34 ± .31	10	< 0.001	7.12 ± .27	10	< 0.001	< 0.01
16	14.49 ± .46	8	< 0.001	10.65 ± .34	8	< 0.001	< 0.001
18	20.36 ± .68	8	< 0.001	15.85 ± .54	8	< 0.001	< 0.001
20	25.28 ± .67	13	< 0.001	16.29 ± .42	12	> 0.5	< 0.001

[1] The probabilities listed in these columns refer to the differences between the means of the preceding line and the line on which the p value appears (within the same category of treatment).

TABLE 2

Fresh weight of muscle (mg per total leg musculature)

DAYS OF INCUBATION	CONTROL (I)			THIOUREA-TREATED (II)			p (I MINUS II)
	Mean ± S. E.	n	p[1]	Mean ± S. E.	n	p[1]	
12	123.7 ± 10.2	6		99.7 ± 11.5	6		> 0.1
14	322.8 ± 21.4	10	< 0.001	297.9 ± 15.2	10	< 0.001	> 0.4
16	552.1 ± 27.2	8	< 0.001	443.9 ± 20.6	8	< 0.001	< 0.01
18	767.3 ± 15.9	8	< 0.001	584.9 ± 12.7	8	< 0.001	< 0.001
20	1,057.44 ± 43.8	14	< 0.001	631.5 ± 31.9	12	< 0.001	< 0.001

[1] The probabilities listed in these columns refer to the differences between the means of the preceding line and the line on which the p values appear (within the same category of treatment).

TABLE 3

Deoxyribonucleic acid content of muscle (mg per total leg musculature)

DAYS OF INCUBATION	CONTROL (I)			THIOUREA-TREATED (II)			p (I MINUS II)
	Mean ± S. E.	n	p[1]	Mean ± S. E.	n	p[1]	
12	.27 ± .02	6		.23 ± .03	5		> 0.1
14	.82 ± .06	8	< 0.001	.74 ± .05	8	< 0.001	> 0.2
16	1.66 ± .10	8	< 0.001	1.33 ± .06	8	< 0.001	< 0.02
18	1.80 ± .10	8	> 0.3	1.66 ± .11	8	< 0.02	> 0.3
20	1.77 ± .06	11	> 0.7	1.69 ± .12	8	> 0.8	> 0.5

[1] The probabilities listed in these columns refer to the differences between the means of the preceding line and the line on which the p values appear (within the same category of treatment).

cates that differences in DNA content between control and experimental embryos exist from the 14th day on. This difference is greatest on the 16th day when the experimental level is approximately 20% lower than the control value. The difference at 16 days is significant at the 2% level, but at all of the other intervals no significance can be demonstrated.

The assumption must be made, therefore, that an alteration in cell or tissue composition must occur and at least at the later stages, be responsible for most of the depression of muscle fresh weight. The ratio of protein nitrogen content to DNA content of the tissue should reveal any alterations in the composition of the nucleocytoplasmic unit in terms of its most significant constituent. The use of this ratio also circumvents the errors that would be introduced were no correction applied to account for the fact that on the 16th day a significant difference exists in the numbers of nuclei in the leg musculature of control and experimental animals. In effect the use of such a ratio averages the cell population and is justified only insofar as it is presented as a basis for comparing experimental and control groups. In figure 2 these ratios are

PROTEIN NITROGEN AND RIBONUCLEIC ACID CONTENT OF MUSCLE
IN CONTROL AND THIOUREA-TREATED EMBRYOS

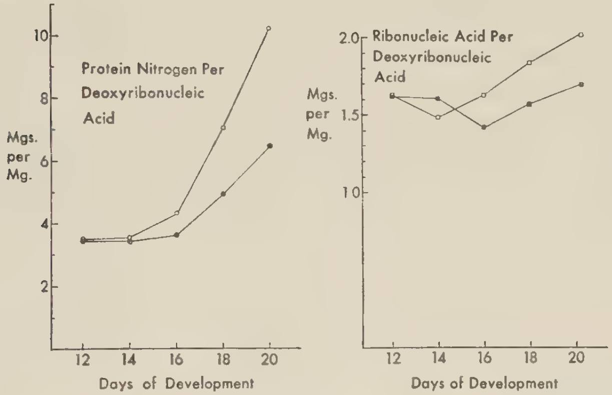

Fig. 2 Open figures represent the means of the control group; closed figures are used to represent the means of the thiourea-treated groups.

plotted against time. The PN/DNA ratio of the experimental group is lower than the control level from the 16th through the 20th day. An examination of the statistical treatment of the linear regression of the PN/DNA ratios given in table 4 indicates that a significant increase in the rate of protein accumulation occurs in control embryos after the 14th day. No corresponding increase in rate occurs in thiourea-treated embryos, however, until after the 16th day. Not only is the initiation of the increase in protein per nucleocytoplasmic unit delayed by thiourea-treatment, but, once initiated the rate of protein accumulation is approximately 52% slower than in control embryos. Comparing the data on total DNA (number of nucleocytoplasmic units) with PN/DNA ratios (protein content per nucleocytoplasmic unit) reveals that decreased protein accumulation per unit rather than a reduction in the number of units is, in fact, the principal mechanism of growth retardation by thiourea administration.

In view of the postulated involvement of ribonucleic acid (RNA) in protein synthesis (Caspersson, '50; Allfrey et al., '53; Brachet, '55; Gale, '55; Spiegleman et al., '55; Webster and Johnson, '56), measurements were made of this muscle component of normal control and thiourea-treated embryos. The data on RNA content are plotted as RNA/DNA ratios in figure 2.

As has been observed in the patterns of protein accumulation, the treatment of linear regression indicates that a significant increase in the rate of accumulation of RNA/DNA also occurs after the 14th day in control embryos, but not until after the 16th day in the thiourea-treated group. Unlike the accumulation patterns of protein, however, once initiated the rate of RNA/DNA accumulation in the thiourea-treated group is not significantly different from the rate of RNA/DNA accumulation in control embryos.

Ample evidence has been presented (Mackenzie and Mackenzie, '43) demonstrating reversibility of the effects of thiourea administration by thyroxine in the post-natal rat. The possbility of differences in permeability (Herrmann et al.,

TABLE 4

Protein nitrogen per deoxyribonucleic acid (PN/DNA — mg/mg)

DAYS OF INCUBATION	CONTROL (I)			THIOUREA-TREATED (II)			p (I MINUS II)
	Mean ± S. E.	n	p[1]	Mean ± S. E.	n	p[1]	
12	3.50 ± .07	6		3.45 ± .16	5		> 0.7
14	3.57 ± .08	8	> 0.5	3.44 ± .05	7	> 0.9	> 0.1
16	4.33 ± .20	8	< 0.01	3.64 ± .18	8	> 0.3	< 0.05
18	7.07 ± .43	8	< 0.001	4.98 ± .12	8	< 0.001	< 0.001
20	10.29 ± .36	11	< 0.001	6.51 ± .24	8	< 0.001	< 0.001

DAYS OF INCUBATION	REGRESSION COEFFICIENT	p[3]	p[1]	REGRESSION COEFFICIENT	p[3]	p[1]	p (I MINUS II)
12–14	0.044	> 0.4		0.007	> 0.9		> 0.6
14–16[2]	0.381	< 0.01	< 0.02	0.100	> 0.3	> 0.3	< 0.05 ($t = 2.069$)
16–20	1.495	< 0.001	< 0.001	0.717	< 0.001	< 0.001	< 0.001

[1] The probabilities listed in these columns refer to the differences between the means of the preceding line and the line on which the p value appears (within the same category of treatment).

[2] The probability of the difference between the means of the regression lines for both groups, calculated from an analysis of variance of joint regression, is smaller than 0.001.

[3] Probabilities of departure from a slope of zero.

'56) and sensitivity, however, necessitates an examination of this question in embryonic tissues. The total dosages of thyroxine were varied from 1 µg to 2.70 µg. In assaying the optimal dose, muscle fresh weight and body weight alone were used as criteria of reversal to facilitate the handling of the relatively large numbers of embryos required. A preliminary study was undertaken using an injection schedule starting on the 12th and terminating on the 18th day with a 48-hour interval between injections. This series indicated a lower survival at a total dose of 2 µg, whereas survival after a total dose of 1 µg was close to 100%. A dosage of 2 µg proved to be the highest practical dose for this series in terms of the numbers of survivors.

Survival was improved by using a schedule with a 24-hour interval between successive injections. Mortalities seemed to occur predominantly after the first and last injections with only a negligible number of deaths between these two times. By starting the series of injections on the 13th day, survival past the initial period of mortality was greately enhanced. Using this revised schedule it was possible to use a total dose as large as 2.35 µg. Survival after a total dose of 2.70 µg was achieved by omitting the 18th-day injection and sacrificing the embryo at that time. In figure 3 the percentage reversal achieved in these cases is plotted against the total dose that would have been administered had the experiment been terminated on the 20th day. Both the 2.35 and 2.70 µg doses, judging from their relative ineffectiveness and toxicity, fall in the thyrotoxic range.

Figure 3 is a concentration curve representing all of the reversal data described above. The data are expressed as percent reversal where the muscle fresh weight of embryos receiving thiourea with no further treatment (or volumes of distilled water equal to the volumes of thyroxine solution administered) is considered to be 0%. The muscle fresh

TABLE 5

Ribonucleic acid per deoxyribonucleic acid (RNA/DNA — mg/mg)

DAYS OF INCUBATION	CONTROL (I)			THIOUREA-TREATED (II)			p (I MINUS II)
	Mean ± S. E.	n	p [1]	Mean ± S. E.	n	p [1]	
12	1.63 ± 0.06	7	> 0.1	1.62 ± 0.08	6		> 0.3
14	1.49 ± 0.07	6		1.61 ± 0.06	7	< 0.05	> 0.2
16	1.63 ± 0.06	8	< 0.001	1.42 ± 0.05	8		< 0.02
18	1.84 ± 0.13	5		1.57 ± 0.08	5	< 0.001	> 0.05
20	2.02 ± 0.10	7		1.70 ± 0.05	7		< 0.01

DAYS OF INCUBATION	REGRESSION COEFFICIENT	p [2]	(III–V)	DAYS OF INCUBATION	REGRESSION COEFFICIENT	p [2]	(IV–VI)	p
12–14 (III)	— 0.0758	> 0.1	$p < 0.01$	14–16 (IV)	— 0.0971	< 0.02	$p < 0.001$	III MINUS IV > 0.7
14–20 (V)	0.096	< 0.01		16–20 (VI)	0.073	< 0.001		V MINUS VI > 0.4

[1] The probabilities listed in these columns refer to the differences between the means indicated by the ends of the braces.

[2] Probabilities of departure from a slope of zero.

The probability of the difference between the means of the regression lines for both groups from the 16th through the 20th day, calculated from an analysis of variance of joint regression, is smaller than 0.001.

weight of control embryos is considered to be 100% "reversed."

$$\frac{\text{FW Reversed} - \text{FW Thiourea-Inj.}}{\text{FW Control} - \text{FW Thiourea-Inj.}} \times 100 = \%\ \text{Reversed}$$

(Where FW equals fresh weight.)

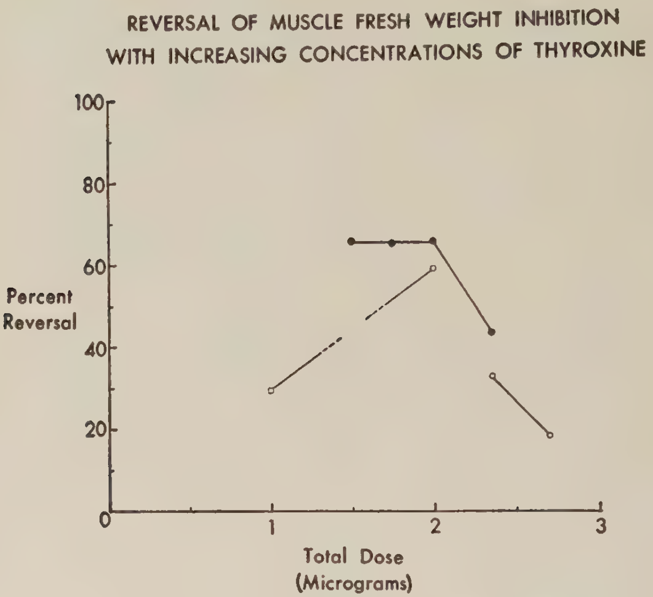

Fig. 3 Open squares represent means obtained with a schedule employing a 48 hour interval between successive injections. The closed circles denote the use of a 24 hour interval between successive injections. The open circles represent means obtained with a 24 hour interval schedule in which the final (18th day) dose was omitted and the embryos sacrificed on the 18th day. Percentage reversal is, in this case, calculated with reference to the 18-day values for control and thiourea-treated groups. The total dose indicated is the amount that would have been administered were it possible to carry this series to completion.

The results obtained with both the 24-hour and 48-hour interval schedules are plotted. Maximal reversal is achieved with total doses of from 1.5 to 2.0 μg. At these doses the deficit in fresh weight accumulation is reversed to approximately 65% of normal. No significant differences can be discerned between the mean reversed levels achieved with a total dose of 1.5, 1.75, or 2.00 μg. Fresh weight achieved with these dosages do differ significantly from the zero control level (thiourea alone). They also differ significantly from the 1.00 μg level, which appears to be below the quantity required for optimal reversal, and the 2.35 μg level, which is apparently in the thyrotoxic range.

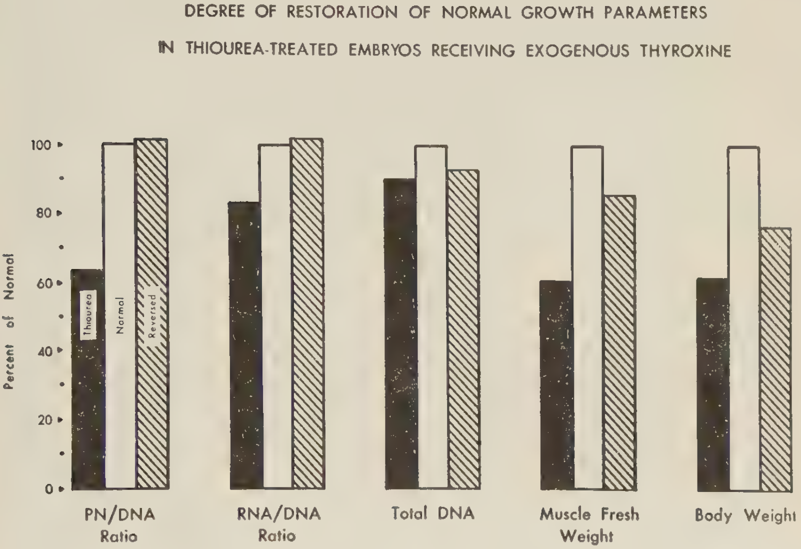

Fig. 4 In each case the black bar represents the thiourea-treated group which received no hormone supplement, the white bar represents the control (100%) level and the shaded bar the percentage obtained by administering, to thiourea-treated embryos, a total of either 1.75 or 2.00 μg of thyroxine.

Despite the fact that the deficit in fresh weight accumulation can be reversed to only 65% of normal, PN/DNA and RNA/DNA ratios show at least 100% reversal (see fig. 4). The statistical treatment in table 6 shows that there is no significant difference between "reversed" and normal control figures except for body weight and muscle fresh weight. Neither is there any significant difference between the "unreversed" group and previously run thiourea-treated groups.

The most likely explanation of this disparity between the reversibility of PN/DNA and RNA/DNA ratios and the

TABLE 6

Degree of restoration of normal growth parameters in thiourea-treated embryos receiving exogenous thyroxine (1.75 to 2.00 µg)

	UNITS	THIOUREA-TREATED [1] (I)	THIOUREA-TREATED PLUS THYROXINE REPLACEMENT [1] (II)	COMPARISON OF II MINUS I		COMPARISON OF NORMAL CONTROLS [2] MINUS II	
		Mean ± S. E.	Mean ± S. E.	% Diff.	p	% Diff.	p
Body wt.	gm	15.57 ± 0.73	19.37 ± 0.42	19.6	< 0.001	23.4	< 0.001
Muscle wt.	mg	635.6 ± 35.4	906.6 ± 25.4	29.9	< 0.001	14.3	< 0.01
Total DNA	mg	1.59 ± 0.06	1.65 ± 0.04	3.6	> 0.4	6.8	> 0.7
PN/DNA	mg/mg	6.53 ± 0.97	10.51 ± 0.45	37.9	< 0.01	—9.6	> 0.8
RNA/DNA	mg/mg	1.68 ± 0.05	2.05 ± 0.06	18.0	< 0.001	—1.5	> 0.9

[1] The means represent averages of 16 cases for group II and 9 cases for group I.

[2] See tables 1 to 5.

fresh weight is suggested by a comparison of the total DNA in "reversed" and normal control groups. Most probably the administration of thyroxine does not reverse the inhibition of DNA accumulation attendant upon thiourea administration. Although the differences in DNA content are small, they could account for the loss of a considerable amount of fresh weight.

Statistical treatment is rendered difficult by the small DNA differences involved. Neither the differences between the control and thiourea-treated groups nor between the controls and the "reversed" group are statistically significant.

The thesis that the decrease in DNA is due to a direct effect of thiourea on muscle tissue and is not mediated though the thyroid has been tested in another fashion. If this were true a second injection of thiourea should result in a greater loss of DNA than that achieved with a single injection. From table 7 it can be seen that a second injection of 7.6 mg of thiourea does, in fact, result in a larger percentage difference below the normal control level at 20 days ($p < 0.001$). The difference between the means of the doubly injected and previously run singly injected groups is significant at the 5% level. The control values in the two types of experiments are not significantly different. Although the DNA levels respond to a second injection of thiourea, neither PN/DNA nor RNA/DNA of the doubly injected group differ significantly from the values of these ratios observed in singly injected embryos. These parameters, then, are regulated by a thyroid-mediated effect which is already maximal after the single injection of thiourea given on the 10th day. The efficacy of the dose of thiourea given at the 15th day was tested by injecting it into embryos which had previously received only a control injection on the 10th day. In this group, too, a significant difference from the total DNA of the control group is observed. Moreover, a significant difference ($p < 0.03$) in PN/DNA ratio occurs between control and 15th day injected embryos. The fact that the RNA/DNA ratio is not affected by an injection on the 15th day may indicate that

TABLE 7

Comparison of the growth parameters of 20-day embryos receiving injections of thiourea on both the 10th and 15th day with those injected once on either the 10th or 15th day

	UNITS	I Control[1]	II Single injection 15th day[1]	III Injected on both 10th and 15th days[1]	IV Comparison of I minus II		V Comparison of I minus III		VI Comparison of single injection on day 10 with respective controls[2]	VII Comparison of controls of column VI with I	VIII Comparison of exptls. of column VI with III
	Units	***Mean + S. E.***	***Mean + S. E.***	***Mean + S. E.***	*% Diff.*	*p*	*% Diff.*	*p*	*% Diff.*	*p*	*p*
Body wt.	gm	23.07 ± 0.67	17.83 ± 0.58	14.44 ± 0.69	22.7	< 0.001	37.4	< 0.001	35.6	< 0.05	< 0.05
Muscle wt.	mg	1,016.7 ± 30.9	703.3 ± 40.4	558.3 ± 37.7	30.8	< 0.001	45.1	< 0.001	40.3	> 0.4	> 0.1
Total DNA	mg	1.90 ± 0.09	1.55 ± 0.07	1.39 ± 0.05	18.4	< 0.01	26.8	< 0.001	4.5	> 0.2	< 0.05
PN/DNA	mg/mg	9.96 ± 0.33	8.30 ± 0.60	6.22 ± 0.32	16.7	< 0.03	37.6	< 0.001	36.7	> 0.5	> 0.4
RNA/DNA	mg/mg	2.11 ± 0.10	2.10 ± 0.15	1.76 ± 0.11	.5	> 0.9	16.6	< 0.05	15.8	> 0.9	> 0.9

[1] The means represent averages of 10 cases for group I, 11 cases for group II and 8 cases for group III.

[2] See tables 1 to 5.

this parameter is amenable to experimental alteration only prior to the 15th day, that is, prior to the time when RNA/DNA starts to rise normally. This would be in agreement with the observation that the rate of increase of RNA/DNA is not affected by thiourea-treatment. However tempting this conclusion may be, it must be held in abeyance on statistical grounds. If the same percentage decrease in absolute RNA/DNA ratios occurred after the single 15th day injection as occurs after a single injection at the 10th day, the mean of the 15-day group would have been 1.96 mg/mg. The magnitude of the difference between this theoretical value and the value actually observed is precisely the standard error of the observed mean.

The percentage reversal of body weight is also represented in figure 4. The degree of reversal of the deficit in body weight is approximately 40% of the uncorrected maximum in contrast to 65% for muscle fresh weight. These data might indicate either a greater irreversible loss of cells or a smaller degree of the restoration of normal cell composition in some other component of body weight with this schedule of thyroxine replacement. However, it is equally likely that it merely indicates a higher fresh weight per cell in organs other than leg musculature. An irreversible loss of the same percentage of cells as in muscle tissue would, thus, involve a greater accompanying loss of fresh weight.

It should be emphasized that although it has been possible to restore certain parameters of muscle growth to the normal level, the replacement procedures employed here cannot be regarded as entirely physiological. This is evident from the protocol alone. However, experimental confirmation is also available: by the 20th day of development, retraction of the yolk sac has occurred in all of the normal control animals. This has never been observed in the thiourea-treated group nor in the "reversed" groups.

DISCUSSION

Thiourea treatment inhibits the growth of the cells of embryonic skeletal muscle by reducing the accumulation of protein per nucleocytoplasmic unit. Since the normal level of protein per nucleocytoplasmic unit can be restored by thyroxine replacement, there is little doubt that the defect is due primarily to a reduction in the amount of circulating thyroid hormone. The temporal characteristics of the response to thiourea administration, however, suggest that a considerable time lag occurs between the onset of function in the embryonic thyroid gland and the involvement of thyroid hormone in protein accumulation in skeletal muscle cells.

Although thiourea is administered as early as the 10th day of development, no clear-cut, thyroid-mediated effect on skeletal muscle can be detected until after the 14th day. Follicular colloid can be demonstrated in thyroids from 10-day embryos (Hopkins, '35) and the incorporation of radioactive iodine into thyroxine has been observed in embryonic thyroids of the same stage of development (Blanquet et al., '53; Trunnell and Wade, '55). The lag in the manifestation of the effect of thiourea treatment is not, therefore, an expression of the state of maturation of the embryonic thyroid gland. Neither does the time lag appear to depend on the rate of development of the goitrogenic effect, since depletion of follicular colloid can be observed as early as the 12th day of development in thyroids from thiourea-treated embryos. Therefore, it would appear that the time course of the response to thiourea administration is a property of embryonic muscle tissue itself. Either thyroid hormone has no influence on muscle metabolism during the lag period or the processes regulated by the hormone may not be critical for protein accumulation during the earlier period.

Recent *in vitro* studies on mitochondrial preparations suggest that the classical interpretation of thyroid hormone action (viz., the regulation of energy metabolism) may be explicable in terms of a regulatory function in oxidative phosphorylation (Martius and Hess, '51; Lardy and Maley, '54; Mudd et al., '55; Tapley et al., '55; Klemperer, '55). This viewpoint is not, however, universally accepted (Barker, '55; Hechter, '55). Irrespective of the mechanism, if the primary effect of thyroid inhibition in the chick embryo is the reduction of the cellular energy level, a curtailment of protein accumulation might be expected as either or both direct and indirect consequences of energy limitation. In this context the lag period may indicate that the energy-generating pathways during this period are insensitive to thyroxine. Thyroxine-insensitive oxidative pathways have, in fact, been demonstrated in certain adult mammalian tissues (Barker and Klitgaard, '52). Nor can we assume that thyroid hormone or the active form of the hormone is at all times available, in sufficiently high concentration, at its site of action (see Gross and Pitt-Rivers, '54; Larson et al., '55). It is equally likely, however, that some other component of the protein-synthesizing mechanism, other than the energy supply, is the limiting factor during the apparent refractory period. Even in the absence of thyroid hormone the energy level may still be high enough to satisfy the requirements imposed by the rate-limiting component of the protein-synthesizing mechanism. Under these conditions alterations in the energy-supplying system would not affect the rate of protein accumulation until the magnitude of this limiting component reached non-limiting levels.

In the sequence of events leading from simpler precursors to the finished protein molecule one could imagine that any one of several components could conceivably limit the rate of synthesis. The only one of these components for which we have measurements at present is ribonucleic acid. The disadvantages of using ribonucleic acid content alone, without attempting intra-cellular localization are self-evident (Siekevitz, '52). However, despite these limitations a suggestive correlation can be seen between ribonucleic acid accumulation and protein accumulation. While both of these cell constituents start to increase simultaneously (per nucleocytoplasmic unit) in normal embryos on the 14th day of development, thiourea treatment delays these increases in both constituents until the 16th day. It is possible that ribonucleic acid is the rate-limiting component suggested above.

However, since thiourea treatment affects both the time of initiation and the rate of PN/DNA accumulation, but only the time of initiation of RNA/DNA accumulation, it seems unlikely that the effect on protein accumulation is completely secondary to an effect on RNA accumulation per se throughout the experimental period. In addition to limitation of the energy supply as a possible factor affecting protein accumulation at the later stages (16th to 20th day) the dynamic state of ribonucleic acid itself must be considered.

The suggestion has been made that a basic difference may exist between established protein synthetic processes and newly-forming pathways in their dependence upon the synthesis of new RNA molecules (Mazia and Prescott, '55). Such a postulation is particularly cogent to embryological problems. The rates of RNA accumulation are not significantly different in experimental and control groups after the 16th day (see table 5). Assuming a dependence on RNA content alone there should be no differences in the rates of protein accumulation in the two groups. Such a difference does exist, however, which may indicate that a greater dependence upon RNA synthesis rather than on accumulation alone may exist after the 16th day. (The correspondence of accumulation rates of RNA in the two groups might merely indicate that the same ratio of rates of synthesis to rates of breakdown is maintained.)

Since it is well known that thyroid hormone is involved in the activity of other endocrine organs (Eartly and Leblond, '54), the problem of the delay in RNA accumulation is being approached from this perspective. The use of hypophysectomized embryos (Fugo, '40) should eventually permit some decision as to the extent of involvement of other endocrine organs in the phenomena described (DiStefano et al., '55; Kingsbury et al., '55).

Aside from the quantitative differences in DNA content between control and experimental embryos, which appear to be consequences of a direct effect of thiourea, the accumulation pattern of DNA in the thiourea-treated group differs from the control. In figure 1 a log plot of DNA accumulation has been included inasmuch as accumulation of DNA by nuclear proliferation would be expected to be an exponential function. Assuming that RNA accumulation is exponential during the period from the 12th to the 14th day, the rate of accumulation decreases in control embryos after the 14th day and comes virtually to a halt after the 16th day. In the experimental group although the rate of accumulation also starts to decrease after the 14th day, and in fact decreases, at first, more rapidly, accumulation continues for a longer period than in the control group. The net result is that the deficit in DNA content observed on the 16th day is compensated for by the 18th day. This restoration of muscle DNA content may either reflect the establishment of a maximum number of nuclei characteristic of this tissue or may be indicative of some degree of hormonal regulation of the duration of the period of DNA accumulation. The later possibility is supported by the accumulation pattern of DNA in the leg musculature of hypophysectomized embryos (Love and Konigsberg, '58).

SUMMARY

1. After the intravenous administration of 8.4 mg of thiourea to chick embryos at the 10th day of development, histological evidence of goitrogenic effects on the thyroid gland can be demonstrated as early as the 12th day of development.

2. No effect on protein or ribonucleic acid content (per unit DNA) of the leg musculature is evident, however, until the 16th day of development.

3. Increases in PN/DNA and RNA/DNA which are initiated on the 14th day in control embryos are delayed until the 16th day of development in thiourea-treated embryos.

4. The rate of protein accumulation in thiourea-treated embryos is 52% lower than the rate in control embryos during the period from the 16th day to the 20th day.

5. Despite the delay in the initiation of the increases in RNA/DNA in the thiourea-treated group, once RNA/DNA starts to rise it accumulates at a rate not significantly different from the control group.

6. A second injection of 7.6 mg of thiourea on the 15th day of development into embryos which had received the initial treatment elicited greater deficits in total DNA content of muscle but produced no further depression of PN/DNA and RNA/DNA than those achieved with the initial single injection alone.

7. A regime of daily intravenous injections of crystalline thyroxine during which a total quantity of either 1.75 or 2.00 μg were administered per embryo completely prevented the deficits in PN/DNA and RNA/DNA but was only partially effective in preventing the decreases in muscle fresh weight and body weight.

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PLATE 1

EXPLANATION OF FIGURES

All of the sections are Bouin-fixed and stained with Schiff's leucofuchsin after periodic acid oxidation (McManus, '48).

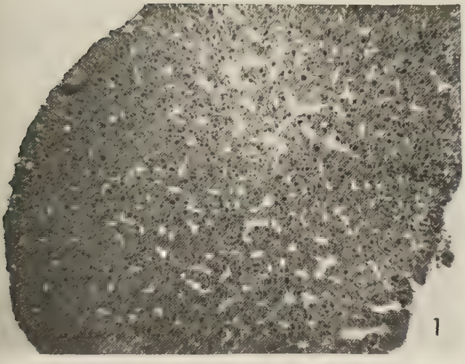
1 Section of thyroid from a control embryo on the 12th day of development. × 200.

2 Section of thyroid from an embryo which had received 8.4 mg of thiourea on the 10th day and was sacrificed on the 12th day. Note the decreased distribution of the deeply staining colloid. × 200.

3 and 4 High power magnification of sections 1 and 2 respectively. Note the significant reduction of the amount of colloid per follicle in the thiourea-treated thyroid (4). × 860.

5 Section of thyroid from a control embryo on the 20th day of development. Note follicles completely filled with deeply staining colloid. × 860.

6 Section of thyroid from an embryo which had received 8.4 mg of thiourea on 10th day and was sacrificed on the 20th day. Note the decreased amount of colloid and the heightened follicular epithelium. (Parathyroid tissue can be seen in the upper right corner.) × 860.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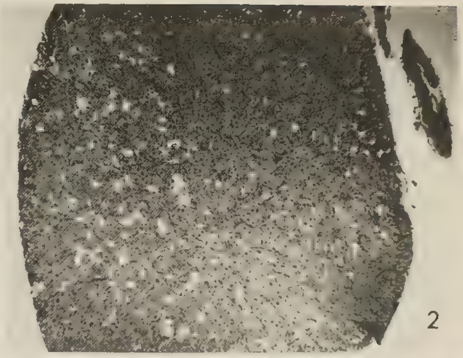
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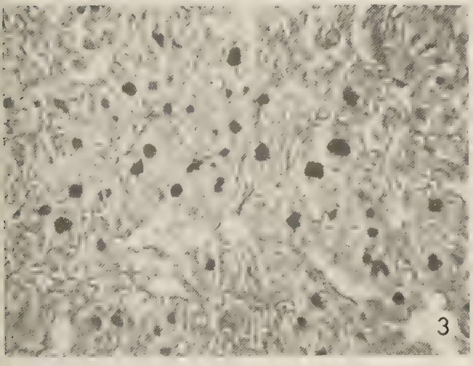
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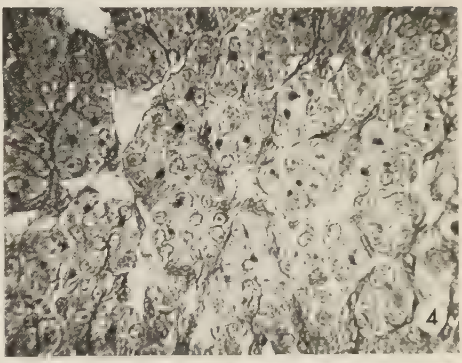
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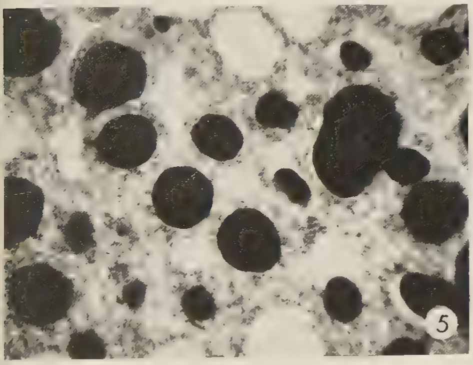
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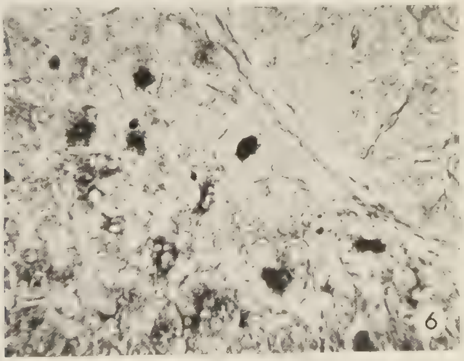
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THE METABOLIC CHARACTERISTICS OF YEAST CELLS TREATED WITH FLUOROACETIC ACID: EVIDENCE OF THE REGULATION OF GLUCOSE OXIDATION BY pH

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INTRODUCTION

In a previous communication (Aldous and Rozee, '56), it was shown that when yeast cells were exposed to fluoroacetic acid (HFA), and then washed free of the inhibitor, the resulting inhibition of oxygen consumption in a 5% glucose medium, depended upon the pH of the medium in which the cells were placed. Thus, at pH 2.1 the poisoned cells showed a 70% inhibition of oxygen consumption whereas at pH 5.0 the same cells consumed oxygen at the same rate as the controls; upon returning the cells to the more acid environment, the inhibition reappeared.

In interpreting these results it was suggested that a change in environmental pH from the acid to the more neutral reaction was responsible for either (a), a dissociation of an enzyme inhibitor complex or (b), a change in the metabolic pathway of glucose associated with the uptake of oxygen.

Evidence points to the Krebs cycle as being the site of inhibition by HFA where, either the conversion of HFA to fluorocitrate results in an inactivation of aconitase (Peters, '52), or an inhibition of the condensing enzyme takes place due to the formation of fluoroacetyl Coenzyme A (Brady, '55). In either case function of the cycle is decreased. Acetate penetrates the intact yeast cell in both the undissociated and the ionic form (Conway and Downey, '50), and since this substrate is believed to be oxidized via the Krebs cycle

(Lynen, '42, '43), we have used acetate oxidation as a criterion of the operational efficiency of the cycle. That the Krebs cycle is present in yeast has been demonstrated by Foulkes ('51).

In view of the above evidence we have assumed as the basis for a working hypothesis that if poisoning involves a metabolic uptake of HFA, then the fact that a cell is poisoned indicates that the Krebs cycle must have been operative during the period of exposure to HFA. Secondly, we have assumed that when a decrease in metabolic function results from an exposure to HFA, the site of this inhibition is the Krebs cycle. While other interpretations of our data are no doubt possible, it seems needless to invoke mechanisms other than those which are currently accepted unless these are proven to be untenable.

Two distinct lines of investigation have been followed in the work to be reported. The first method involves an examination of the environmental conditions under which HFA poisoned cells exhibit a decrease in metabolic function, i.e., the behavior of poisoned cells in environments of varying pH or substrate. The second entails a study of the environmental conditions which induce HFA uptake, i.e., the conditions under which poisoning takes place. If the working hypothesis is correct, it is evident that the information given by one line of investigation must be substantiated by the other.

MATERIALS AND METHODS

The test organism used throughout was Fleischman's bakers' yeast, which was obtained locally in cake form.

Fed cells. After washing thoroughly in M/20 KH_2PO_4 to remove the starch "filler," the cells were made up as a 1% v/v suspension on M/20 KH_2PO_4 containing 5% glucose, and were aerated for two hours. For use, the cells were centrifuged and resuspended in buffer of desired pH to which acetate or glucose was added.

Starved cells. Washed cells were suspended in KH_2PO_4 (M/20), and were aerated for two hours. Previously performed experiments had shown this procedure to reduce the cellular metabolism to the endogenous level without too great a depletion of the reserve metabolite (s). For use, the cells were centrifuged and suspended in buffer of desired pH.

Buffer. Throughout all the experiments reported, McIlvaine's citric acid-phosphate buffer was used since the pH range from 2.2 to 7.0 can be covered with this one mixture.

Some experiments were done to check on the possibility that a variation in pH might induce a change in respiratory characteristics because of a change in the concentration of the ions in the buffer. Cells in which the poisoning, washing, and resuspending procedures were all carried out in phosphoric acid-sodium phosphate buffer exhibited the same respiratory characteristics as those in which McIlvaine's buffer had been used.

As the latter buffer maintained a more constant pH, especially in the acid range, it was used throughout the experiments to be reported.

Glucose-buffer. This refers to McIlvaine's buffer containing 5% glucose.

Acetate-buffer. Acetic acid was added to McIlvaine's buffer and the pH adjusted to the desired value.

HFA Pure sodium fluoroacetate (Bios Laboratories, Inc.) was dissolved in buffer, and the pH adjusted to the required value.

Treatment with HFA. To a 1% suspension of cells, HFA was added to give a final concentration of 5×10^{-3} M. After 30 minutes shaking at 25°C, the cells were centrifuged, washed twice with buffer of the same pH as that at which exposure was made, and were finally resuspended in buffer with or without the addition of substrate. In all experiments, control cell suspensions, to which buffer instead of HFA was added, were shaken and put through identical washing procedures.

The rate of oxygen consumption was measured at 25°C by the direct method of Warburg. The values reported in the tables and figure 1, represent the averaged readings for one hour after a steady state had been reached.

The fluid volume of each vessel was 2 ml made up entirely of yeast cells suspended in the substrate-buffer mixture. The amount of respiring material was approximately 18 mg wet weight, or 3.5 mg dry weight per vessel.

The pH of the solutions was always checked at the end of an experiment to make sure no change from the original had taken place.

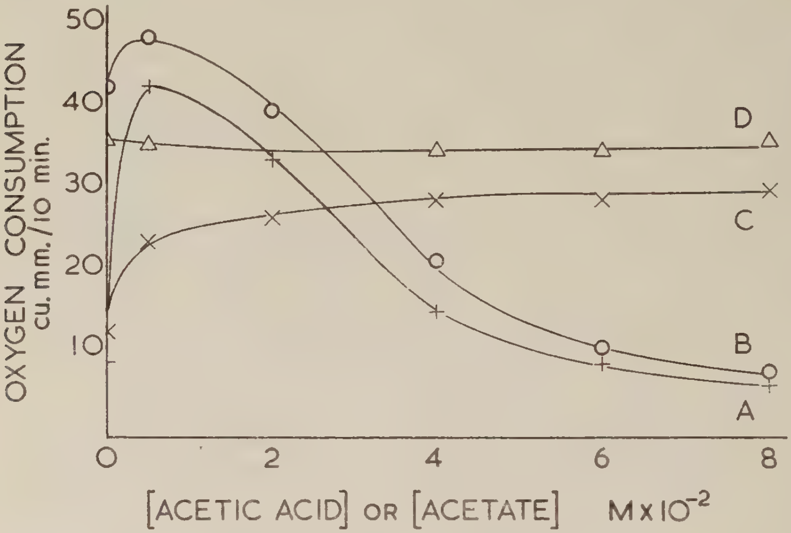

Fig. 1 Oxygen consumption of fed yeast cells as a function of the concentration of undissociated acetic acid or ionized acetate in the environment and the effect of these concentrations upon the subseuqent utilization of glucose. Curve A, buffer pH 2.7 containing acetic acid in concentrations indicated on abscissa. Curve B, the same, one hour later, after the addition of glucose to give a final concentration of 1%. Curve C, same as A, but at pH 6.7 (ionized acetate). Curve D, same as B, but as pH 6.7.

RESULTS

The effect of pH upon the respiration of normal cells in acetate-containing media

Before investigating the ability of the HFA-poisoned cell to utilize acetate under the conditions of pH which would be demanded, it was necessary to establish the effect of pH upon the acetate oxidation of normal cells. Although the literature contains information on this problem, the facts are not at all clear, especially in view of the general principle that undissociated molecules usually penetrate cells more readily than the corresponding ions.

Both Tang and Wu ('38), and Barron, Ardao and Hearon ('50), showed that yeast cells oxidize acetate at progressively higher rates as the pH of the solution approaches the pKa of acetic acid from the acid side. These data are contrary to theoretical expectations since they suggest that acetate is oxidized more effectively in the ionized form. Acetate has been shown to inhibit glucose oxidation (Tang and Wu, '38), fermentation (Maesen, '53), and phosphate uptake (Sampson, Katz and Harris, '55). These inhibitions show the same type of variation with pH, namely a progressive decrease as the pH is varied from the acid to the alkaline side of the pKa of acetic acid. Since some of the above references, (Maesen, '53; Samson, Katz and Harris, '55), contain data indicating that these inhibitions are also a function of acetate concentration, an alternate interpretation of the effect of pH on acetate oxidation suggested itself. The fall in rate of acetate oxidation with decreasing pH observed by Barron et al., ('50), might well be explained on the basis of the appearance of the inhibitory acetic acid molecule rather than to the disappearance of the oxidizable acetate ion. Undissociated acetic acid might thus be visualized as exerting an inhibitory action on its own oxidation perhaps through an oversaturation effect.

In order to test the ability of the normal cell to oxidize either undissociated acetic acid or ionized acetate, in various concentrations, fed cells were suspended in acetate-buffer at pH 2.7 and at 6.7, wherein the concentration of the substrate was varied from 0.5 to 8×10^{-2} M. The rate of oxygen consumption was then followed for one hour. At this time glucose, to make a 1% solution, was tipped from the side arm into the main space of the vessel in order to observe the effect of acetate upon the oxidation of glucose. The results of one such experiment are shown in figure 1, where the rate of oxygen consumption is plotted against acetate concentration. The rate of the blank, or endogenous metabolism is indicated by the symbols for curves A, and C, plotted at zero on the concentration axis. From these data it is clearly seen (a), that the rate of oxygen consumption (curve A) decreases with increasing concentration of undissociated acetic acid, and this effect is reflected in the rate at which glucose is oxidized during the second hour of the experiment, (curve B). Thus the rate of oxygen consumption in a mixture of the two substrates is determined by the concentration of acetic acid in the mixture. (b), In ionized acetate (curve C), the rate of oxygen consumption is virtually independent of acetate concentration until values less than 2×10^{-2} M are reached. When glucose is added to the medium (curve D), the rate attained is independent of acetate concentration, and is the same as that observed in glucose alone (zero on the concentration axis). The latter results would suggest that in a mixture of acetate and glucose, the latter substrate is utilized in preference to the former, and thus entirely corroborate the findings of Eaton and Klein ('54), in this respect.

The observation that at 5×10^{-3} M, the lowest concentration used, the rate of respiration induced by undissociated acetic acid is much greater than that attained in ionized acetate, points to a more ready penetration of the cell by the undissociated form of the substrate.

These observations are pertinent to the problem under consideration because of the likelihood that the cell does not distinguish between fluoroacetate and acetate.

The respiratory characteristics of cells poisoned in glucose-containing media

In order to observe the effect of HFA poisoning on acetate oxidation, fed cells were treated with HFA at pH 2.2 in the presence of 5% glucose. After being washed, the cells were placed in acetate-buffer at pH 2.2 and 6.7. The rate of oxygen consumption of control and treated cells is recorded in table 1.

The data indicate that exposure to HFA results in a reduction in the ability to oxidize acetate which is almost identical at the two extremes of pH. *Thus the metabolic block induced by HFA is not reversed by changing the environmental pH,* and the phenomenon previously described therefore cannot be associated with the effect of pH on the metabolic inhibitor.

TABLE 1

The respiratory characteristics of cells exposed to HFA at pH 2.2 in a glucose medium

Exposure conditions: Fed cells in 5% glucose-buffer, pH 2.2 exposed to 5 × 10^{-3} M HFA for 30 minutes; washed twice.

Respiring conditions: 0.01 M acetate-buffer, pH 2.2 and 6.7. Cells allowed to shake in thermostat for one hour before measurements made.
Temperature 25°C.

pH	OXYGEN CONSUMPTION (MM^3 PER 15 MINS.)		INHIBITION
	Control	Treated	
			%
2.2	45.2 ± 1.06 [1]	7.07 ± 0.14	84.3
6.7	24.4 ± 0.10	5.02 ± 0.08	79.4

[1] Standard error of mean.

It would seem to be a necessary conclusion that the disappearance of the inhibition of glucose oxidation above a pH of about 4.5 (Aldous and Rozee, '56) is due to a shift in the metabolic pathway of the cell. If the metabolic block resulting from an exposure to HFA occurs at the citrate stage in the Krebs cycle, and the inhibition of glucose oxidation in poisoned cells is absent at pH 6.7, it follows that at this pH oxidation of glucose must be proceeding by a pathway which either does not involve citrate, or which is distinct from the cycle. In either case it seems reasonable to presume that the Krebs cycle does not operate under these particular conditions. From this assumption it follows that is should not be possible to poison cells with HFA in the presence of glucose at pH 6.7, because the reactions concerned with the metabolic uptake of HFA are not in operation. The ability to oxidize acetate or glucose should therefore be virtually normal.

Table 2, containing the data from an experiment designed to test these suggestions, shows the expectations to be essentially correct, although a small inhibition of acetate oxidation is apparent where none is expected. This inhibition is to be compared to that of some 80% when exposure was carried out at pH 2.2 (table 1). The results in table 2, might, however, be accounted for on the basis of the impenetrability of the fluoroacetyl ion, a possibility which was subsequently tested.

TABLE 2

The respiratory characteristics of cells exposed to HFA at pH 6.7 in a glucose medium

Exposure conditions: Fed cells in 5% glucose-buffer, pH 6.7 exposed to 5 × 10^{-3} M HFA for 30 minutes; washed twice.

Respiring conditions: 5% glucose-buffer pH 2.2 and 6.7 or 0.01 M acetate-buffer pH 2.2 and 6.7.

Temperature 25°C.

SUBSTRATE	pH	OXYGEN CONSUMPTION (MM ^{3} PER 15 MINS.) CONTROL	OXYGEN CONSUMPTION (MM ^{3} PER 15 MINS.) TREATED	INHIBITION
				%
Acetate	2.2	77.3 ± 3.40[1]	66.7 ± 2.14	13.5[2]
Acetate	6.7	57.6 ± 4.03	49.7 ± 5.05[3]	0
Glucose	2.2	71.6 ± 5.78	74.5 ± 4.16[3]	0
Glucose	6.7	71.7 ± 4.53	66.8 ± 3.99[3]	0

[1] Standard error of mean.

[2] $p < 0.05, > 0.01$.

[3] Not significantly different from control.

The respiratory characteristics of cells poisoned in acetate-containing media

The data of table 1, and figure 1, indicate that non-poisoned cells oxidize acetate almost as readily at pH 6.7 as at pH 2.2, and if this substrate is metabolized through the Krebs cycle, then it should be possible to poison cells with HFA at pH 6.7 by substituting acetate for glucose in the experiment of table 2.

In order to be sure that during exposure to HFA, the cells would be actively metabolizing acetate, and not previously-assimilated products of glucose metabolism, suspensions of starved cells were used in the experiments of this series. Exposure to HFA was made in acetate-buffer, pH 6.7, for the same length of time as previously used. After washing, the cells were placed in either acetate-buffer or glucose-buffer at pH 2.2 and 6.7. The observed rates of oxygen consumption are recorded in table 3.

TABLE 3

The respiratory characteristics of cells exposed to HFA at pH 6.7 in an acetate medium

Exposure conditions: Starved cells in 0.005 M acetate-buffer, pH 6.7 exposed to 5 × 10^{-3} M HFA for 30 minutes; washed twice.

Respiring conditions: 5% glucose-buffer pH 2.2 and 6.7 or 0.01 M acetate-buffer pH 2.2 and 6.7.

Temperature 25°C.

SUBSTRATE	pH	OXYGEN CONSUMPTION (MM^3 PER 15 MINS.)		INHIBITION
		CONTROL	TREATED	
				%
Acetate	2.2	42.1 ± 1.72 [1]	7.9 ± 0.65	81.2 [2]
Acetate	6.7	41.8 ± 2.22	6.3 ± 0.65	84.9 [2]
Glucose	2.2	45.9 ± 1.02	27.2 ± 0.29	40.7
Glucose	6.7	54.2 ± 1.41	47.9 ± 0.23	11.3

[1] Standard error of mean.

[2] Not significantly different.

When these results are compared with those of table 2, it is apparent that substituting acetate for glucose during treatment results in an uptake of HFA by the cell; failure of the ionized HFA to penetrate the cell cannot, therefore, be invoked as an explanation of the data of table 2.

From the above observations it also follows that if exposure to HFA is made in an acetate medium of low pH, cells with the same qualitative characteristics as those in table 3 should be obtained. That such is the case may be seen in table 4, where the cells were exposed to HFA for 30 minutes in acetate-buffer, but at pH 2.2 instead of 6.7.

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The data in tables 3 and 4, are qualitatively consistent with the hypothesis advanced earlier both with respect to the behavior of poisoned cells toward pH and substrate, and in terms of the effect of these factors on the ability to poison cells. This hypothesis states that in order to poison cells with HFA, environmental factors must initiate operation of the Krebs cycle, and, in addition, that once the cells have been poisoned by HFA, the effect of the metabolic block will only be seen when environmental factors favor Krebs cycle operation. Quantitative differences in glucose metabolism do appear, however, when the cells have been poisoned in an acetate medium. Our earlier observations (Aldous and Rozee, '56), and those of table 2, would lead us to expect the inhibition of glucose oxidation in tables 3 and 4, to be about 70% at pH 2.2, and zero at pH 6.7. Thus an exposure of cells to acetate in some manner brings about small but significant changes in the subsequent metabolism of glucose.

TABLE 4

The respiratory characteristics of cells exposed to HFA at pH 2.2 in an acetate medium

Exposure conditions: Starved cells in 0.005 M acetate-buffer, pH 2.2 exposed to 5×10^{-3} M HFA for 30 minutes; washed twice.

Respiring conditions: 5% glucose-buffer pH 2.2 and 6.7 or 0.01 M acetate-buffer pH 2.2 and 6.7.

Temperature 25°C.

SUBSTRATE	pH	OXYGEN CONSUMPTION (MM ^{3} PER 15 MINS.) CONTROL	OXYGEN CONSUMPTION (MM ^{3} PER 15 MINS.) TREATED	INHIBITION
				%
Acetate	2.2	27.8 ± 0.53 [1]	6.0 ± 0.26	77.8 [2]
Acetate	6.7	41.4 ± 3.55	10.0 ± 0.65	75.8 [1]
Glucose	2.2	39.9 ± 1.71	16.4 ± 0.60	59
Glucose	6.7	56.7 ± 0.90	48.3 ± 1.50	15

[1] Standard error of mean.

[2] Not significantly different.

DISCUSSION

It is perhaps pertinent to point out that the phenomena described have been observed to take place in a medium which must be essentially free of HFA. If HFA penetrates the cell then the fact that the poisoning by HFA persists after two washings is strong evidence that the original inhibitor molecules have undergone a metabolic change to a compound which does not diffuse easily out of the cell, such as might be expected if HFA had been metabolized to fluorocitric acid. A metabolic change of this type is usually associated with enzymes of the Krebs cycle occurring at, or within the mitochondria (Nossal, '53). As these bodies are located in the intracellular environment, buffered effectively at about pH 6.0 (Conway and O'Malley, '46), it is not likely that fluctuations in extracellular pH would influence these reactions directly. Experimental evidence of this is contained in table 1, where it is shown that the inhibition of acetate oxidation in HFA-poisoned cells is virtually unchanged when the environmental pH is varied over a wide range. Tables 3 and 4, show also that acetate is capable of initiating the metabolic uptake of HFA equally well at the two extremes of pH. These observations we would interpret as evidence that acetate provides a substrate for the Krebs cycle in a manner which is independent of environmental pH.

Glucose is also capable of providing substrates for the Krebs cycle as long as the pH of the medium is less than about 4.5. Under these conditions glucose not only initiates poisoning by FHA (Aldous and Rozee, '56, and table 1), but in addition its oxidation is inhibited in those cells which have been exposed to HFA in the presence of acetate (tables 3 and 4), provided that the glucose oxidation is taking place in a medium of low pH. These observations point to the probability that the metabolism of glucose in acid solution involves the same reactions as does acetate metabolism, namely the Krebs cycle.

The alternate route by which glucose oxidation proceeds when the environmental pH is greater than 4.5 is more difficult to identify. Two observations suggest that either the Krebs cycle is completely by-passed, or that acetate does not arise as an intermediate: Table 3 (also Aldous and Rozee, '56), illustrates the fact that poisoned cells are able to oxidize glucose at an almost normal rate whereas their ability to oxidize acetate is greatly reduced. This observation has been interpreted as indicating that glucose does not provide a substrate (perhaps acetate) for the Krebs cycle. In support of this interpretation the data of table 2, shows at pH 6.7, glucose fails to initiate the reactions which lead to the uptake of HFA, and subsequent poisoning of the cell. It should be noted that the Krebs cycle is quite capable of operation at this pH (tables 1–4), but when glucose is present the reactions concerned with the oxidation of acetate cease to function. Additional evidence of this ''inhibitory'' effect of glucose is provided in figure 1, curves C and D. Here the rate of oxygen consumption in a mixture of the two substrates is characteristic of that in glucose alone.

It would appear, therefore, that there is a route of glucose oxidation which does not include acetate as an intermediate. This suggestion has been made by a number of authors working with a variety of micro-organisms. Hirsch ('55), has observed that yeast is capable of carrying on a limited oxidation of glucose despite marked deficiencies in the enzymes of the Krebs cycle. Essentially the same phenomenon has been observed in *E. coli* (Umbarger, '53), and in *P. pestis* (Englesberg, Gibor and Levy, '54; Englesberg, Levy and Gibor, '54). Eaton and Klein ('54) noted that ''young'' yeast cells were capable of actively oxidizing glucose beyond the acetate stage although they were incapable of oxidizing acetate or ethanol due to deficiencies of enzymes in the Krebs cycle. These authors state that this alternate route of glucose oxidation does not involve the hexose-monophosphate shunt.

A noteworthy characteristic of the alternate route is its sensitivity to H^+. As the concentration of H^+ in the environment becomes greater about 10^{-5} molar/the oxidation of glucose proceeds to a progressively lesser extent by this route, and to a correspondingly greater extent by means of the Krebs cycle (Aldous and Rozee, '56). For reasons which have been elaborated earlier, the Krebs cycle does not appear to be directly influenced by environmental pH, therefore, its regulation must be mediated by the effect which pH has upon the alternate route.

The direct influence of environmental pH on the alternate route points to the probability of a reaction at the cell surface being involved. Rothstein ('54), has shown that in a medium devoid of K^+ the rate of glucose fermentation is dependent upon pH. The stimulating action which K^+ has upon the process over the range pH 2.0–4.0, could be differentiated from that occurring over the range pH 5.5–7.5. In the more acid range, K^+ stimulation resulted in an increase in alcoholic fermentation whereas in the more neutral range it was associated with glycerol fermentation. The metabolic shift thus occurs somewhere between pH 4.0 and pH 5.5. It is to be noted that this coincides almost exactly with the range in which the Krebs cycle-alternate route shift has been observed to take place in the present investigation. Rothstein also noted that oxygen consumption was stimulated by K^+ but to a much lesser extent than was carbon dioxide production. The relation between these observations and our findings will form the subject of a future communication from this laboratory.

The fact that conditions exist under which exposure to HFA does not result in a blocking of the Krebs cycle (table 2), may alter the interpretation of data in the literature which have been reported in ignorance of this fact. Samson, Katz and Harris, ('55), noted that HFA did not alter the inhibition of phosphate uptake by acetate. Since they assumed that a block of acetate oxidation had been produced by HFA they interpreted the above observation to indicate that the inhibition of phosphate uptake must have been due to a direct action of acetate, and not to the products of its oxidation. In the light of our findings, two features of the above experiments are worthy of note: (a) the pH of the medium was 5.5, and (b), glucose was also present as an external substrate. Under such conditions one might expect that glucose rather than acetate would be oxidized (as the data of figure 1 suggest), and in addition, that pH would direct the oxidation of glucose largely by way of the alternate route. In other words, in our experience, these are the environmental conditions under which HFA poisoning does not take place to any great extent.

Black and Hutchens ('48), record somewhat similar observations with respect to oxygen consumption induced by various substrates in the presence of HFA. They found that whereas acetate and succinate added alone or together failed to stimulate the oxygen consumption of poisoned yeast cells, the addition of glucose resulted in a much higher rate of respiration. As this work was carried out at pH 4.8, the environmental conditions would favor operation of the alternated route, and would account for the metabolic response to the added glucose.

The occurrence of alternate pathways for glucose oxidation only one of which is sensitive to HFA, does not appear to be confined to yeast. Strauss ('55), used HFA to elucidate the mode of acetate metabolism in *Neurospora crassa*. Tests with a wild type were carried out at pH 4.0, whereas those using an acetate-mutant were done at pH 6.0. Strauss' data, using HFA to block acetate metabolism show a remarkable similarity to the situation reported herein for yeast. A 4×10^{-3} M solution of HFA produced, at pH 4.0, a 68% inhibition of acetate utilization and a 64% inhibition of glucose oxidation in the wild strain. At pH 6.0 the acetate-mutant showed a 71% inhibition of acetate oxidation, but the reduction in glucose oxidation amounted to only 28% despite the fact that the HFA concentration in this case had been raised to 1.2×10^{-2} M. (The fact that a different strain was used does not alter the agrument since acetate was available to the cells).

These observations, together with those cited earlier, suggest that the influence of environmental factors on carbohydrate metabolism of a variety of micro-organisms may follow a somewhat similar pattern.

CONCLUSIONS

Assuming that exposure of yeast cells to HFA results in a blocking of the Krebs cycle, the results of this investigation have demonstrated the following points:

1. Living yeast cells possess two routes for the oxidation of glucose.
2. One of these routes is operative when the environmental pH is < 4.5 and has been tentatively identified as the Krebs cycle. The metabolic function of the Krebs cycle is not directly affected by H^+ ion.
3. At a pH > 4.5 an alternate oxidative pathway is brought into operation at the expense of the Krebs cycle. This route would appear to involve enzymes at the cell surface since it is directly influenced by environmental pH.
4. Poisoning of yeast cells with HFA will only place when environmental conditions initiate operation of the Krebs cycle. Both pH and external substrate are important in determining these conditions.

It has also been shown that undissociated acetic acid serves as an oxidizable substrate in low concentrations, but in high concentrations it acts to inhibit glucose oxidation as well as its own oxidation. The acetate ion possesses no such inhibitory properties.

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THE PRESSURE, TEMPERATURE AND ION RELATIONS OF MYOSIN ATP-ASE [1]

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FIVE FIGURES

The kinetics of the hydrolysis of adenosine triphosphate (ATP) by the muscle protein myosin have received considerable attention in recent years, and several reviews have been devoted to this subject (Szent-Györgyi, '55; Morales et al., '55; Buchthal et al., '56). The ATP-ase activity of myosin is modified by pressure (Laidler and Beardell, '55), by calcium, magnesium and nucleotides (Blum, '55), and by calcium, pH and temperature (Green and Mommaerts, '54; Ouellet et al., '52).

The effects of pressure, temperature and ions on ATP-ase activity have been interpreted in terms of a simple Michaelis-Menten reaction scheme (Laidler and Beardell, '55; Green and Mommaerts, '54), although other studies of the behavior of myosin have implicated a series of reactions (Mommaerts and Hanson, '56; Morales et al., '55; Watanabe et al., '53). In fact, Spicer ('55) has suggested that reversible denaturations may explain gelation phenomena in solutions of myosin B.

Our interest in the problem arose from the observation that pressure reduces the isometric twitch tension of intact muscle at lower temperatures and increases it at higher (Cattell and Edwards, '28). This has been ascribed to the effect of pressure on an activation process in the muscle (Brown, '41). The discovery that pressure also affects the sol-gel equilibrium of myosin (Marsland and Brown, '42) adds interest to the problem. Myosin has a volume about 120 cm^3/mole larger as a gel than as a sol. Such a large volume change often indicates configurational modification of a protein, and configurational modification of myosin might also influence its ATP-ase activity.

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The ATP-ase activity of myosin is altered by pressure, temperature, specific ion and ionic strength effects, and substrate and product concentrations. We have attempted to simplify analysis of the system by working at substrate concentrations high enough to saturate the enzyme, so that the reaction of enzyme with substrate could be assumed to be complete. Preliminary results with this saturated system (Brown and Lawler, '46) showed large effects of pressure on the ATP-ase activity of muscle protein. However, these experiments were made before myosin A and myosin B had been clearly distinguished, and were carried out at the distinctly non-physiological pH of 8.4. The present data extend the earlier study to lower pH and to more clear-cut enzyme preparations.

The influence of pressure on the ATP-ase activity of rabbit myosin A was studied in the presence of excess substrate and of the activator calcium. Pressure increases the activity at high pH and decreases it near pH 6. This indicates that at least two reactions are pressure-sensitive, as indicated in a preliminary report (Guthe et al., '54). The system, at high substrate concentration, may be analyzed in terms of a reversible denaturation and a rate process.

$$\begin{tikzcd} M_n - ATP \arrow[r, "b"'] \arrow[d, shift right, "a"'] & M_n + ADP + P_i \\ M_d \arrow[u, shift right] \end{tikzcd} \qquad (1)$$

where P_i is inorganic phosphate, M_n is native myosin, and M_d is reversibly denatured myosin. An analogous interpretation has been applied to bacterial luminescence, where pressure and temperature influence light production through their effects on a rate process and on the reversible denaturation of luciferase (Brown, Johnson and Marsland, '42; Eyring and Magee, '42; Johnson, Eyring and Polissar, '54).

Temperature or pressure may alter the over-all ATP-ase activity by changing either the equilibrium constant of the reversible denaturation (a) or the velocity constant of the hydrolytic reaction (rate process b). Effects on the formation of the enzyme-substrate complex or of the calcium-enzyme complex are excluded for the following reasons. At high substrate concentration, ATP saturates the active enzymatic sites of myosin, so that neither pressure nor temperature can change the amount of enzyme-ATP complex. The data of the present paper show that the activation of myosin by calcium ion is not pressure-sensitive. This paper is chiefly concerned, therefore, with the establishment of the existence of reversible denaturation in myosin and with the separation of the effects of temperature and pressure on the reversible denaturation from their effect on the rate process.

These effects must be interpreted in terms of absolute reaction rate theory (Glasstone, Laidler and Eyring, '41), which has been applied to many chemical systems as well as to biological reactions (Johnson, Eyring and Polissar, '54). A detailed application of the theory to myosin ATP-ase is presented in another paper (Guthe and Brown, '58).

METHODS

Myosin. Myosin was extracted from ground rabbit skeletal muscle with Weber-Edsall solution (0.5 M KCl buffered with bicarbonate to pH 7.4) for 2½ hours at 0°C. Twenty volumes of water were added to the extract, and the precipitate was washed twice with 10 volumes of buffer. The precipitate was dissolved in 0.5 M KCl. Usually the pH of this solution was adjusted to 6.3. The myosin was reprecipitated with 20 volumes of water, then redissolved in KCl and reprecipitated. This last (third) precipitate was diluted twenty to two hundred-fold with 0.1 M buffer to obtain enzyme activities in the conveniently measurable range. This material will be called precipitated myosin.

In a second method, the initial extract was dialyzed for 12 hours against 0.1 M buffer at pH 7.5, then washed twice with 10 volumes of buffer and packed by centrifugation. The precipitate was then diluted with buffer as in the first procedure. This material will be called dialyzed myosin.

For measurements of the pH-dependence of the pressure effects, myosin was prepared by extraction with an acid phosphate-KCl mixture (Guba-Straub) at pH 6.2. Precipitation, solution and two reprecipitations were carried out as in the first method, but without any washing with buffer.

After the preparations were complete, the precipitated myosin was stored in 0.03 M KCl, the dialyzed myosin in 0.1 M buffer. The more dilute salt solution seems to preserve the enzymatic activity better than 0.5 M KCl, and no activity changes were noted under the present test conditions for the first week. Myosin concentrations were determined by the Kjeldahl method.

Activity measurements. The experiments were carried out in sodium glycinate, cacodylate or veronal[2]-acetate buffers of ionic strengths between 0.1 and 0.14. Myosin is a gel under these conditions. Unstirred solutions split ATP as rapidly as stirred solutions, so that accessibility of the enzyme does not limit the rate of high ATP concentrations. The ATP-ase activity of myosin, free from actin, shows no discontinuity on gel-sol transformation (Mommaerts and Green, '54; Hasselbach, '52).

Activities were measured by the inorganic phosphate produced (Fiske-Subbarow method). Two milliliters of the dilute buffered myosin solution were mixed with 2 ml of buffered ATP and 0.10 ml of $CaCl_2$. At the end of the experiment 4 ml of 10% trichloroacetic acid were added to stop the reaction. The phosphate in a 2-ml aliquot of the mixture was then measured colorimetrically. Experiments were performed in duplicate. After preliminary measurements, an enzyme concentration was chosen such that the phosphate split remained linear with time for 40 minutes, the duration of the experiments.

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[2] Unrecrystallized veronal in the presence of calcium has a deleterious effect on the enzyme. Under pressure, the enzyme is precipitated in the presence of crude veronal and calcium at pH 8.0.

The experiments were made with 2.6 mM ATP, about 10 times the concentration at which the enzyme is saturated with substrate. Experiments with two and three times these concentrations showed little or no inhibitory effect of excess substrate. Excess substrate does not inhibit myosin, free from actin, although it does inhibit actomyosin (Green and Mommaerts, '54).

Pressure equipment. Hydrostatic pressures were developed hydraulically and transmitted to a filled and stoppered test tube suspended in the pressure fluid. The pressure chamber was large enough to hold 4 pairs of test tubes for simultaneous application of pressure.

RESULTS

Hydrogen ion, pressure and myosin ATP-ase

If the effect of pressure on the activity of myosin ATP-ase were a direct effect on the rate constant, the logarithm of the pressure increment should be proportional to pressure. The volume change with which the reaction proceeds could then be determined by comparing the ATP-ase rate (v_p) at a single pressure (p) to the atmospheric rate (v). The rates at 7800 pounds per square inch and at atmospheric pressure depend on pH, as shown in figure 1. The effects of pressure and pH are reversible.

Pressure has opposite effects at neutrality and at high pH. It decreases the activity below pH 7.4 and increases it in more alkaline solution. Apparently pressure affects two different processes, as indicated in reaction scheme (1). We assume that high pH favors reversible denaturation, which pressure opposes. By reactivating denatured myosin, pressure increases the amount of active enzyme and thus the ATP-ase activity. At low pH, on the other hand, relatively few of the enzymatic sites are denatured, and the observed decrease in activity with pressure is attributed to effects on the rate process. Activity at atmospheric pressure increases at higher pH despite increased denaturation, so that the rate process must increase rapidly as the pH increases, perhaps proportionally to the hydroxyl ion concentration, as in Mommaerts' data ('54).

With these assumptions, the effects of pressure on pH on reversible denaturation can be determined if the observed activity under pressure is corrected for the direct inhibition of the rate process. If the inhibition is not pH-dependent, the observed rate may be multiplied by a constant factor, which will bring the observed rate at 7800 psi at pH 6.3 into agreement with the observed rate at atmospheric pressure at pH 6.3. The corrected rates (v'_p) still depend on pH, because both the atmospheric rate and the pressure-sensitive reversible denaturation depend on pH. To remove the former dependence, one turns to the corrected pressure increment, ($v'_p/v - 1$). Values for this function, taken from the smooth curves through the observed points, are plotted against pH in figure 1b, and represent the pH-dependence of reversible denaturation.

The curve of figure 1b is calculated for the dissociation of a single hydrogen ion. It represents the increase in the reversibly denatured form of myosin with increasing pH. Its theoretical justification is considered in another paper (Guthe and Brown, '58). The right-hand curve represents the number of denatured sites at a given pH when the system is under a pressure of 7800 psi, while the left-hand curve represents the larger number of inactive sites at atmospheric pressure. Although the apparent volume changes show the same pH dependence as an acid-base equilibrium, the effect of pressure is much larger than expected for a simple acid dissociation. Extensive changes in protein configuration must also occur.

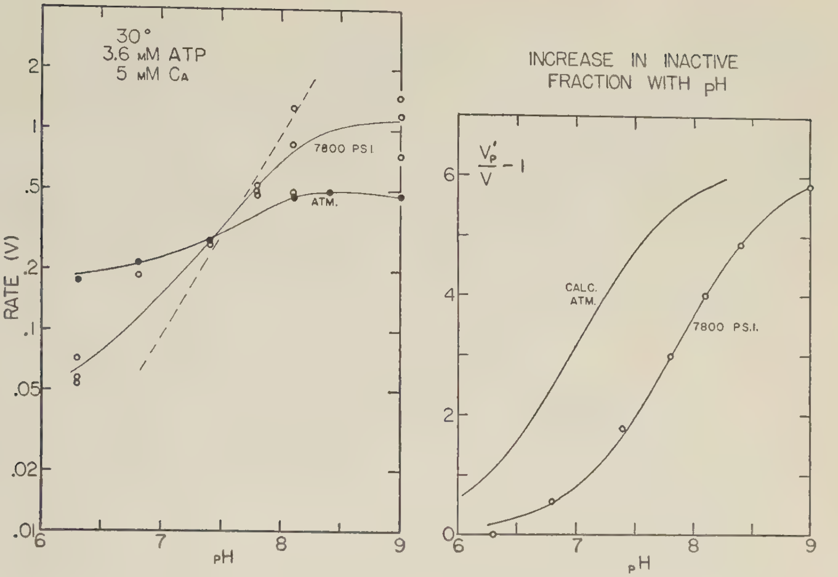


Fig. 1 Left: Dependence of myosin ATP-ase activity on pH at two pressures. The dashed line, which is approached by the data for 7800 psi, corresponds to a direct proportionality between activity and hydroxyl ion concentration.

Right: Corrected relative pressure increment inactivity as a function of pH. V'_p is the observed activity corrected for direct pressure inhibition (see text). Points are derived from the smooth curves of the preceding graph. Curves are theoretical titration curves for a monobasic acid. The abscissa is proportional to the fraction of reversibly denatured sites. The left-hand curve is calculated.

Calcium equilibrium

The data of the preceding section were obtained at constant calcium concentration. The question arises whether these effects reflect a change in the equilibrium constant for the combination of myosin with its known activator calcium. The observed activity depends on calcium concentration as shown in figure 2. Calcium clearly increases the activity of the enzyme, and so do temperature and pressure.

Under identical conditions, different ATP-ase activities were found for different preparations of myosin, as well as for a single preparation at different times. In the calcium experiments, preparation differences were avoided by use of a single preparation, and aging effects were eliminated through the following procedure. At one temperature, the ATP-ase activities of myosin at different calcium concentrations were measured simultaneously. Then other series of measurements were made at other temperatures, until three such series had been obtained. Finally, activities at the different temperatures were measured simultaneously at constant calcium concentration (1.7 mM). All these activities were then reduced to the same basis (fig. 2).

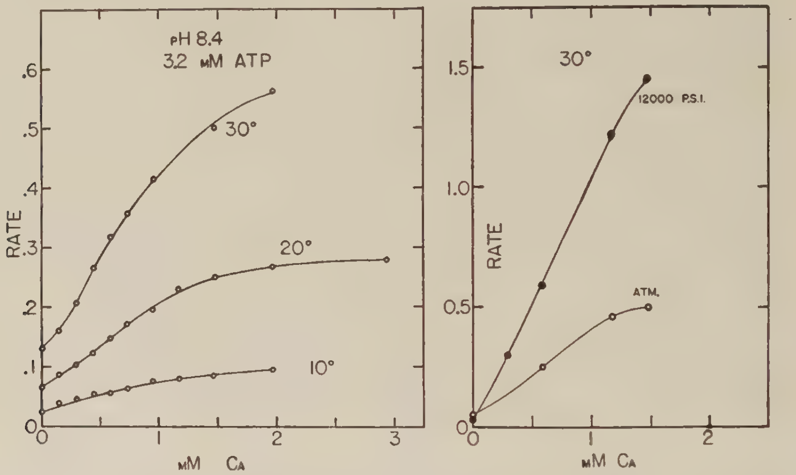


Fig. 2 Dependence of myosin ATP-ase activity on calcium concentration. Left: Activity-concentration curves are the same shape at different temperatures. Right: Activity-concentration curves are the same shape at different pressures.

The curves at the different temperatures are alike in shape. The equilibrium constant for calcium-myosinate dissociation is related to the calcium concentration at which the activity increment has reached half its maximum. This concentration is 0.79 mM for all three temperatures and for both pressures. Although combination with calcium increases the ATP-ase activity of myosin, the equilibrium constant does not depend significantly on temperature or pressure, and the pressure-sensitive equilibrium of the preceding section cannot be the association of calcium with myosin. The ATP-ase activity is also temperature-sensitive, while the calcium equilibrium is not.

Although the major effects of temperature and pressure are not on the calcium equilibrium, the latter must be slightly sensitive to these factors. In the absence of added calcium, pressure decreases myosin ATP-ase activity at any pH. ATP-ase activity under these conditions proceeds with an apparent volume increase of about 20 cm^3/mole. Since the pressure effect is different in the absence of calcium, the calcium effect must depend at least slightly on the pressure.

Low heats of reaction have previously been found for the binding of calcium by other proteins (Katz and Klotz, '53). Since the size of the pressure effect often follows that of temperature, calcium activation of myosin might have been expected to be relatively insensitive to both factors. The present data confirm this expectation.

Although the calcium-myosin equilibrium is not pressure-sensitive, its nature is of interest in its own right. The ATP-ase activity of the myosin at any calcium concentration will be the sum of the activity of calcium-free myosin and the activity of one (or more) calcium-myosin complex. Let v_0 be the activity of the calcium-free myosin and v_m the activity of the calcium-myosin complex in which *m* calcium ions have combined with each active site of myosin.

Then

$$v = v_0(M) + v_m(MCa_m) \qquad (2)$$

where (M) and (MCa_m) are the total concentrations of myosin and of its calcium complex. The total concentration of myosin, whether combined with calcium or not is $(M)_t$, and

$$(M)_t = (M) + (MCa_m) \qquad (3)$$

The equilibrium constant for dissociation of the calcium myosinate is

$$K_m = \frac{(M)(Ca^{++})^m}{(MCa_m)} \qquad (4)$$

Substituting (3) and (4) in (2), the activity is

$$v = \frac{v_0 + v_m \dfrac{(Ca^{++})^m}{(K_m)}}{1 + \dfrac{(Ca^{++})^m}{K_m}} \qquad (5)$$

Equation (5) can be rewritten in two alternative forms:

$$\left(\frac{v}{v_0} - 1\right) = \left(\frac{v_m}{v_0} - 1\right) \frac{(Ca^{++})^m}{K_m + (Ca^{++})^m} \qquad (6)$$

$$\frac{v - v_0}{v_m - v} = \frac{(Ca^{++})^m}{K_m} \qquad (7)$$

Equation (6), if m is unity, becomes formally identical to the Michaelis-Menton equation, but is not otherwise interesting in the present discussion. Equation (7), on the other hand, shows that when log $\left(\frac{v - v_0}{v_m - v}\right)$ is plotted against log (Ca^{++}), one should find a constant slope equal to m, the number of calcium ions bound at each enzymatic site. Figure 3 shows that m is two for the experiments, as suggested on other grounds by Hansl ('56). K_m is the value of $(Ca^{++})^2$ at which $\left(\frac{v - v_0}{v_m - v}\right)$ is one, or 6.2×10^{-7} (moles/liter)^2.[3]

That m is two suggests that two calcium ions are bound at each enzymatic site, but other explanations are possible. This m is analogous to the n of Hill's equation for the oxygenation of hemaglobin, which measures an interaction between active sites. Interaction may be present here, or an enzyme-substrate-activator (ESA) complex may be formed by the combination of monocalcium myosin complex with monocalcium ATP complex (di Stefano and Neumann, '53). The concentration of ESA complex would then depend on the square of the calcium concentration.

Temperature and activity

The effects of temperature on ATP hydrolysis have not yet been considered. Temperature does not significantly affect

[3] Manganese also activates myosin ATP-ase, as previously reported by Bowen ('52) for the ATP-ase of myosin B threads. At its maximum, it produces about one-third the activity that calcium does. The manganese complex is not only less active but less stable than the calcium complex. Its dissociation constant is 3.6×10^{-6} (moles/liter)^2.

the calcium equilibrium and the formation of the enzyme substrate complex should not be rate-limiting at high substrate concentrations. The effects of temperature on the system must then be due to its effects on reversible denaturation and

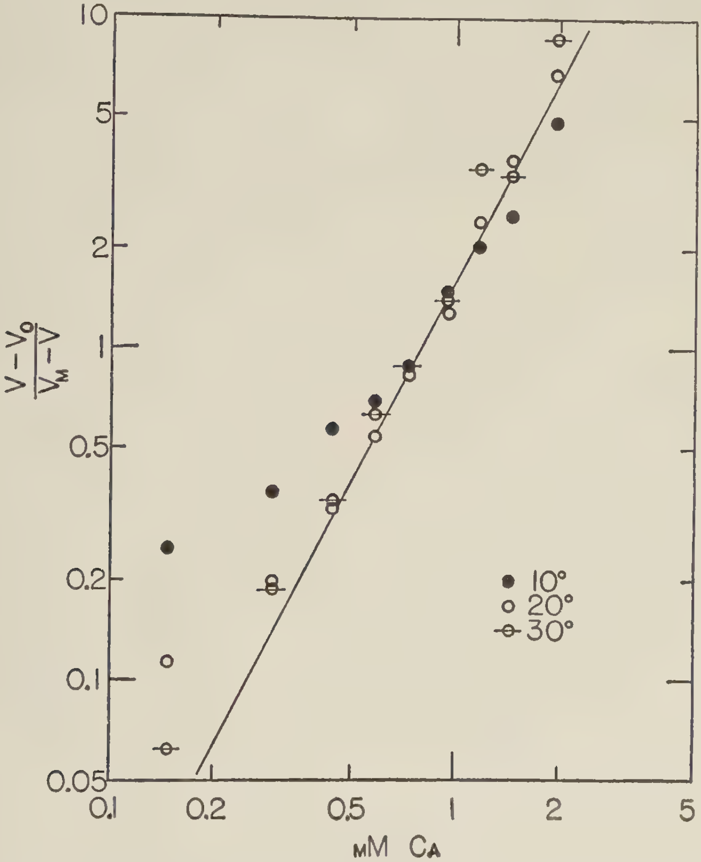


Fig. 3 Analysis of the activity: calcium data of figure 2 in accordance with equation (7). The line has a slope of two, indicating that the concentration of active enzymatic sites depends on the square of the calcium concentration.

on the rate process. The observed activities at different temperatures, for dialyzed myosin, are shown in figure 4a. Activities under a pressure of 9000 psi were also measured at different temperatures (fig. 4b).

At 30°C, the activities of different dialyzed myosin preparations varied from 0.25 to 0.69 μM phosphate/mg-min. in glycine buffer of pH 8.4. Experiments were made at several temperatures on one preparation to obtain the correct relative activities. Activities were then arbitrarily adjusted to 0.51 at 30°C before plotting. If the effect of temperature is interpreted as that on a simple rate process, the activation energy is 18,000—20,000 calories per mole, depending on just where the line is drawn. A somewhat lower value was found for precipitated myosin, 12,400 calories per mole, as reported in a preliminary note (Guthe et al., '54) and as found by Ouellet et al. ('52) for their myosin B preparations. Dialyzed myosin may be less subject to reversible denaturation than precipitated, and denaturation may be more than a single-step process. Further work is needed to explain this difference.

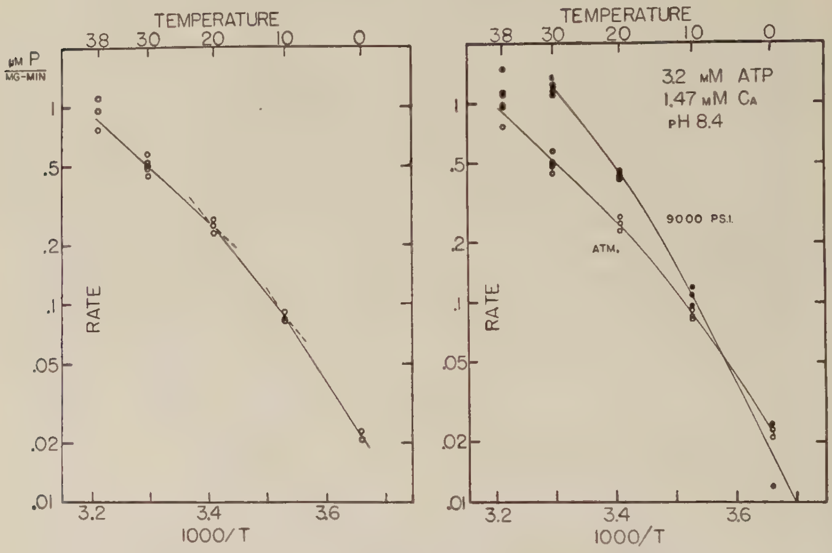


Fig. 4 Dependence of myosin ATP-ase activity on temperature. Left: Activities at atmospheric pressure. Heats calculated for successive straight-line portions of the curve decrease with increasing temperature. Right: Activities at atmospheric pressure and at 9,000 psi.

The temperature dependence of the pressure effect is consistent with the earlier discussion of its pH dependence. Increasing temperature favors reversible denaturation, which pressure opposes. Pressure accordingly increases the activity more at higher temperatures, where reversible denaturation has become more important. The observed activity therefore increases with temperature because the rate of enzyme-substrate breakdown has a high energy of activation. The increase is reduced to some extent because reversible denaturation decreases the number of active enzymatic sites. This second effect is smaller than the first, and the over-all temperature dependence closely approaches linearity. The relatively small heat of reversible denaturation contrasts with the larger one found for bioluminescence.

The small pressure effect at low temperatures has two possible explanations. Either denaturation is appreciable even at low temperatures, or denaturation is negligible and enzyme-substrate breakdown proceeds with a much smaller volume change at 0°C than at 30°C. The second alternative is favored by the results. At 0°, pressure has little or no effect at any pH, so that the volume changes for both denaturation and the rate process must be small at this temperature. The volume changes must therefore depend on temperature, as they do in the bacterial luminescence system (Eyring and Magee, '42).

Pressure and activity

It remains to be shown what part the two reactions play in the dependence of activity on pressure. If pressure directly affects a rate process, log v_p/v should be proportional to pressure. Proportionality is found at pressures under 8000 psi (fig. 5), but the activity increases more slowly as the pressure rises further, and it eventually reaches a plateau. The proportionality at low pressures corresponds to an apparent volume decrease on activation of 44cm^3/mole, as previously reported (Guthe et al., '54). However, this is again the result of opposite effects on two processes, for the existence of a plateau is clear evidence that pressure affects an equilibrium process. Although increasing pressure brings in more of the renatured enzyme, this effect reaches a limit when reversal of denaturation is essentially complete. The effect of pressure on a pure rate process has no limit. The curve therefore shows the presence of both an equilibrium, in which pressure favors the active ATP-ase, and of a rate process, which is either unaffected or inhibited by pressure. These data confirm the assumption that the pressure-favored, neutral-pH form of myosin is the native enzyme. The curve, calculated on the basis of the proposed scheme, fits the experimental points.

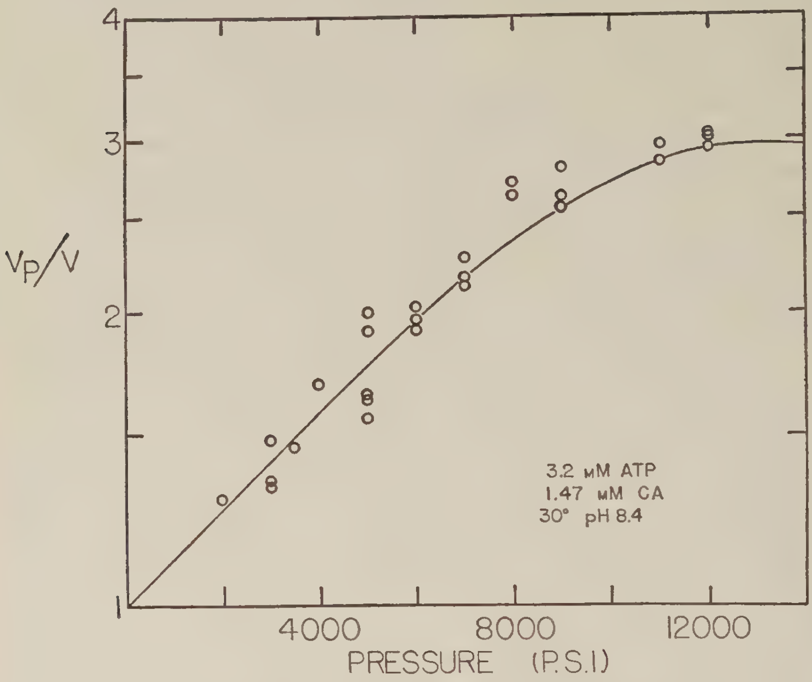


Fig. 5 Pressure increment in myosin ATP-ase activity as a function of pressure. For discussion of data points see text. The curve is the calculated increment for a two-fold pressure effect, on enzyme-substrate breakdown and on reversible denaturation.

It should be noted that pressure increases the activity of some myosin preparations more than others, and that v_p/v was arbitrarily brought to 3.0 at 12,000 psi. This value was observed in most experiments, although some ranged as low as 2.5. Values at intermediate pressures were corrected on a percentage basis.

The variation of the pressure effect with the preparation in itself supports the present reaction scheme. If pressure directly affected only a reaction rate constant, the observed variation would mean that the volume of activation changed from preparation to preparation. According to the present scheme, it is the extent of initial reversible denaturation that varies with the preparation; there may be slight differences in pH or in the concentration of other components of the solution. On the other hand, denaturation itself may not be pH-dependent, but the denatured form may be a stronger acid than the native.

$$\begin{array}{l} \mathrm{HM_n}^{\mathrm{ATP}} \longrightarrow \mathrm{HM_n} + \mathrm{ADP} + \mathrm{P_i} \\ \;\downarrow\!\uparrow \\ \mathrm{HM_d} \rightleftharpoons \mathrm{H}^+ + \mathrm{M_d} \end{array} \qquad (8)$$

If such a two-step reaction occurs, the difference in pressure effect might depend on the experimental conditions and preparative methods through the first equilibrium.

If the complexities of the reaction system are disregarded, and the activity: pressure curve considered linear, we obtain an apparent volume decrease on activation of 44 cm^3/mole at pH 8.4 and 30°C for myosin A. Laidler and Beardell ('55) found a decrease of 32 cm^3/mole at pH 7 and 25°C for myosin B. All our preparations of myosin A show an apparent volume increase at pH 7 and 20–30°, but preparations of myosin B show reasonable agreement with Laidler and Beardell.

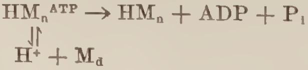
DISCUSSION

The familiar analysis of luminescence in terms of absolute reaction rate theory (Johnson, Eyring and Polissar, '54) has been followed in this paper. The detailed numerical analysis is presented elsewhere (Guthe and Brown, '58). It is appropriate here, however, to review the argument for the occurrence of reversible denaturation in the myosin ATP-ase system.

The opposite effects of pressure on the ATP-ase activity at pH 6.3 and 8.4 show that two different reactions are pressure-sensitive. The equilibrium nature of one reaction is indicated both by pressure: pH data and by activity: pressure data. This equilibrium behaves like a reversible denaturation, with the denatured form favored by increased pH and the native form by increased pressure. If reversible denaturation is instantaneous, pressure increases ATP-ase activity by increasing the concentration of native enzyme through the equilibrium. At neutral pH, nearly all the enzyme is native, and pressure decreases ATP-ase activity by its direct effect on the rate constant for the breakdown of enzyme-substrate complex, which therefore must proceed with an increase in volume.

The pressure: pH data also show that reversible denaturation proceeds with the loss of a single hydrogen ion. The question arises whether the pressure-sensitive equilibrium is properly a reversible denaturation, especially since the equilibrium is much less sensitive to temperature than most reversible denaturations. However, the system is much too pressure-sensitive for a simple hydrogen ion dissociation, and the large volume change for the equilibrium is typical of reversible denaturation. The equilibrium can perhaps be compared to alkaline rather than heat denaturation.

The pressure-sensitive equilibrium is not an activator-enzyme equilibrium, for the activation of the ATP-ase by calcium ion is independent or only slightly dependent on pressure and temperature. It cannot be the enzyme-substrate equilibrium, because the enzyme is saturated at the present high substrate concentrations. The temperature and pressure dependence of activity at a single pH is consistent with the proposed two-fold effect of pressure, and with the following reaction scheme.



$$\begin{array}{l} HM_n^{ATP} \rightarrow HM_n + ADP + P_i \\ \quad \downarrow\!\uparrow \\ \; H^+ + M_d \end{array} \qquad (9)$$

The reversible denaturation may have been overlooked by previous workers because it is most evident in pressure: pH data, which have not previously been reported. It is also possible that our use of sodium buffers, rather than the customary potassium buffers, has increased the role of reversible denaturation in our experiments.

In any event, it is of interest that the myosin molecule is capable of a reversible configurational change that is sensitive to the mechanical variable pressure. Whether this particular pressure-sensitive change is in part responsible for the pressure-sensitivity of the twitch of an intact muscle cannot be decided without further investigation.

SUMMARY

The myosin-ATP-ase system must include at least 4 reactions. One of these is substrate-sensitive, one is calcium-sensitive, and two are independent of these factors but sensitive to temperature, pressure and pH. At high ATP and calcium ion concentrations, the relation between ATP-ase activity and pressure depends on the last two reactions: the reversible inactivation of the enzyme by pH, and the breakdown of the enzyme-substrate complex. Near pH 6, pressure decreases the ATP-ase activity, chiefly because it slows the breakdown, while above pH 8.4, pressure increases the activity by reactivating pH-inactivated enzymatic sites. At intermediate pH, both processes are important.

The action of pressure shows that both reactions proceed with an increase in volume and that pressure, by reducing the volume, slows the breakdown of the complex but favors the native over the denatured enzyme. The extent of denaturation may be related to the presence of sodium ions in our reaction mixtures instead of the customary potassium ions.

Combination of the enzyme with its activator, calcium, is not sensitive to temperature and pressure. The calcium-myosin equilibrium is therefore characterized by a very small energy and volume of reaction. According to kinetic data at pH 8.4, the active calcium-myosin complex may contain two

calcium ions per active site, with an overall dissociation constant of 6.2×10^{-7} (moles/liter)^2.

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REVERSIBLE DENATURATION IN THE MYOSIN ADENOSINE TRIPHOSPHATASE SYSTEM[1]

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ONE FIGURE

The influence of temperature, pressure and pH on the adenosine triphosphatase (ATPase) activity of myosin as set forth in the paper by Brown et al. ('58) is very similar to their influence on bacterial luminescence (Brown, Johnson and Marsland, '42). The satisfactory application of the theory of absolute reaction rates to the luminescence data (Eyring and Magee, '42) suggests its application to the myosin data.

According to this theory, a rate process is in fact an unstable equilibrium between an activated complex and its reactants (Glasstone, Laidler and Eyring, '41; Johnson, Eyring and Polissar, '54). The equilibrium constant for this equilibrium is customarily represented by the symbol K*. The specific reaction rate constant, k′, is

$$k' = \kappa \frac{kT}{h} K^* \qquad (1)$$

in which κ = transmission coefficient
k = Boltzmann constant
h = Planck's constant
T = absolute temperature

The transmission coefficient can usually be taken as unity, and the term kT/h has the numerical value 5.56×10^{12} at 0°C.

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Like any ordinary equilibrium constant, K^* can be related to thermodynamic values for free energy, heat, entropy and volume. Thus the effect of pressure on a rate process depends on the difference in volume between the activated complex and its reactants.

Brown et al. ('58) have shown that the following reactions occur in the myosin ATPase system:

(a) $M + ATP \rightleftharpoons MATP$
(b) $M + 2\,Ca \rightleftharpoons MCa_2$
(c) $HM_nATP \rightleftharpoons HM_nATP^* \rightarrow HM_n + ADP + P_i$
(d) $HM_n \rightleftharpoons M_d + H^+$,

where M is myosin, M_n is native myosin, M_d is reversibly denatured myosin, ATP is adenosine triphosphate, ADP is adenosine diphosphate, P_i is inorganic phosphate, and * denotes the activated complex. It is not known whether denatured myosin combines with calcium ion and ATP or not. In any event, only (c) and (d) are concerned with the response of the system to pressure, temperature and pH under the experimental conditions of Brown et al. Reaction (a) was driven strongly to the right by the use of very high substrate concentrations, and reaction (b) was shown to be insensitive to temperature and pressure.

Myosin ATPase activity (v) is measured by determining the rate of increase of inorganic phosphate:

$$v = \frac{\partial(P_i)}{\partial t} = k'_c(HM_nATP), \qquad (2)$$

where parentheses denote concentration. At high substrate concentration, (HM_nATP) is very nearly equal to the total amount of active enzyme (HM_n),

$$(HM_nATP) \simeq (HM_n)\ . \qquad (3)$$

For reaction (d),

$$K_d = \frac{(H^+)(M_d)}{(HM_n)}\ . \qquad (4)$$

The total amount of native and inactivated enzyme (M_t), is constant for any set of experiments, and is given by

$$(M_t) = (HM_n) + (M_d) = (HM_n) + \frac{K_d}{(H^+)}(HM_n), \qquad (5)$$

so that

$$(HM_n) = \frac{(M_t)}{1 + \frac{K_d}{(H^+)}} . \qquad (6)$$

Substituting (6) in (3) and then (3) in (2), we have for the activity

$$v = k'_c \frac{(M_t)}{1 + \frac{K_d}{(H^+)}} . \qquad (7)$$

Qualitatively, it is clear that when (H^+) is much larger than K_d (when the equilibrium favors the native myosin) the observed rate of hydrolysis for a fixed amount of myosin will depend only on the specific rate constant for (c). The observations at pH 6.3 show that pressure decreases this rate constant.

On the other hand, at higher pH the equilibrium becomes more and more favorable to the inactive myosin. Since the activity at atmospheric pressure increases with pH, the specific rate constant for (c) must also be pH sensitive, increasing with pH so rapidly that it masks the effect of inactivation. Later in this analysis, it will be shown that, as Mommaerts ('54) has already suggested, k'_c increases in direct proportion to the hydroxyl ion concentration. Although pressure decreases the rate constant k'_c, it increases the measured activity v. Equilibrium (d) must therefore also be pressure-sensitive, pressure favoring the native form. Since pressure *increases* the activity at alkaline pH, more than enough denatured enzyme must be reactivated to outweigh the pressure inhibition per active site, or, in other words, the volume change in the equilibrium must be larger than that in the rate process.

As in the luminescence analysis, one can replace k_c and K_d by

$$k'_c = k''_c \frac{kT}{h} e^{-\frac{\Delta E^* + p\Delta V^* - T\Delta S^*}{RT}} \qquad (8)$$

and

$$K_d = e^{-\frac{\Delta E + p\Delta V - T\Delta S}{RT}} , \qquad (9)$$

where k''_c still varies with pH, although its variation with temperature and pressure has been removed. Then (7) becomes

$$v = \frac{\left\{ k''_c \frac{k}{h} e^{\frac{\Delta S^*}{R}} \right\} T e^{-\frac{\Delta E^* + p\Delta V^*}{RT}}}{1 + \frac{1}{(H^+)} e^{-\frac{\Delta E + p\Delta V - T\Delta S}{RT}}} = \frac{BTe^{-\frac{\Delta E^* + p\Delta V^*}{RT}}}{1 + \frac{1}{(H^+)} e^{-\frac{\Delta E + p\Delta V - T\Delta S}{RT}}}. \qquad (10)$$

In this equation, B in the final expression replaces the bracket, which contains unknown factors that are independent of temperature and pressure but still pH-dependent.

At low pH, where a negligible fraction of the myosin is denatured, (10) becomes

$$v = BTe^{-\frac{\Delta E^* + p\Delta V^*}{RT}}. \qquad (11)$$

The ratio of the activity v_p at pressure p to the activity v at atmospheric pressure at the same temperature is then

$$v_p/v = e^{-\frac{p\Delta V^*}{RT}}. \qquad (12)$$

The difference in volume between the activated complex and the reactants can therefore be determined from the extent of inhibition of activity by pressure at pH 6.3. The activity at this pH under 7800 psi is 31% of the activity at atmospheric pressure so that the volume of the activated complex is 56 cm^3/mole larger than the volume of the reactants.

At higher pH, where the effect of reversible denaturation cannot be neglected, the ratio v_p/v at constant temperature and pH is

$$v_p/v = e^{-\frac{p\Delta V^*}{RT}} \frac{1 + \frac{1}{(H^+)} e^{-\frac{\Delta E - T\Delta S}{RT}}}{1 + \frac{1}{(H^+)} e^{-\frac{\Delta E + p\Delta V - T\Delta S}{RT}}}. \qquad (13)$$

It is convenient to define $v'_p = v_p/e^{-(p\Delta V^*/RT)}$, and to note that the equilibrium constant for reversible denaturation at pressure p is

$$K_p = e^{-\frac{\Delta E + p\Delta V - T\Delta S}{RT}}$$

With these substitutions, equation (13) becomes

$$v'_p/v = \frac{1 + \frac{K_p}{(H^+)}\left(e^{\frac{p\Delta V}{RT}}\right)}{1 + \frac{K_p}{(H^+)}} = \frac{(H^+) + K_p e^{\frac{p\Delta V}{RT}}}{(H^+) + K_p}. \qquad (14)$$

To estimate K_p and ΔV, the volume difference between native and denatured myosin, it is convenient to use the corrected pressure increment

$$v'_p/v - 1 = \frac{K_p}{(H^+) + K_p}\left(e^{p\Delta V/RT} - 1\right). \qquad (15)$$

Brown et al. include a plot of this increment against pH and the shape of their curve is consistent with equation (15); the numerical values obtained from the graph are $pK_p = 7.85$ at 7800 psi and $\Delta V = 97$ cm^3/mole, the denatured form having the larger volume. Reversible denaturation, then, proceeds with a large increase in volume. When ΔV is known, the pK for the dissociating hydrogen ion at atmospheric pressure can be calculated, and it is 6.96. The size of the volume increase shows that reaction (d) is more than a simple acid-base equilibrium. The reaction must involve extensive configurational changes as well, and can therefore properly be termed a reversible denaturation. It is probably more than coincidence that a very similar volume change is found for the gelation of myosin, the gel form, like the denatured form, having the larger volume.

At this point in the analysis it becomes possible to show the pH-dependence of the activity of a native enzymatic site. From the equilibrium constant, one can calculate the fraction of the sites inactivated at each pH. The activity per active site ($BTe^{-(\Delta E/RT)}$) is then determined by dividing the observed activity by the fraction active. The result is shown in figure 1. The straight line in the figure, which fits the points well above pH 7, has a slope of one. The rate then is inversely proportional to the first power of the hydrogen ion concentration, or directly proportional to the hydroxyl ion concentration. This relationship was found by Mommaerts ('54) for myosin ATPase between pH 7 and 9 in 0.15M KCl. In the present data, activities below pH 7 are larger than the linear relation predicts. Perhaps another reaction enters below pH 7, as is indicated by the large peak in activity that Mommaerts finds at pH 6.2 in 0.15M KCl.

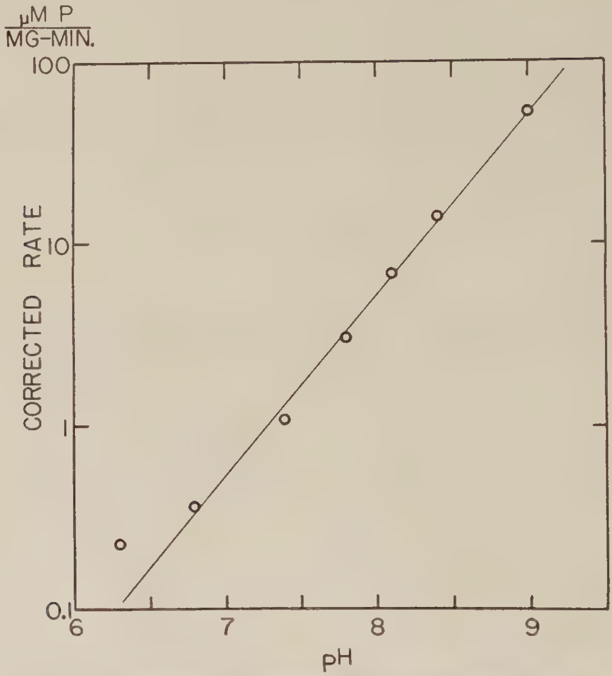

Fig. 1 The pH dependence of the rate of ATP hydrolysis per *active* site, after correction for the fraction reversibly denatured (see text).

Another possible explanation of the data might be that two active forms of enzyme were present, a more active alkaline form and a less active acid form. If this were so, calculation shows that pressure would decrease the rate constant for the acid form, increase that for the alkaline form to the same extent, and favor the acid form in the equilibrium between the two. This is not a very satisfying result, and although the scheme fits the pH data satisfactorily, it fails to account for the observed shape of the activity: pressure curve (fig. 5 of Brown et al.) More complicated reaction schemes could be assumed in which hydrolysis of ATP is catalyzed by either H^+ or OH^- in the presence of the enzyme, while the enzyme is reversibly denatured by either acid or alkaline pH. It seems premature to present an extended analysis based on a more complicated scheme without more extensive data.

At present, the pH dependence of the ATPase activity is left undefined, as part of term B in equation (10), and we proceed to the other thermodynamic constants. Although it may now seem easy to evaluate the energies and the entropy, there is an added complication. The volume changes were determined at a single temperature, but they are temperature-dependent, as are the volume changes for both rate and equilibrium constants in bioluminescence (Eyring and Magee, '42). Activities at 0°C, although less precisely determined than the much larger activities at 30°C, show clearly that pressure has little if any effect at any pH. If the volume changes at 0°C are taken as zero, within experimental error, the volume changes depend on temperature as follows:

$$\begin{aligned} \Delta V^* &= -483 + 1.77\,T \\ \Delta V &= -882 + 3.23\,T. \end{aligned} \tag{17}$$

The corresponding relations for luminescence (Eyring and Magee, '42) are

$$\begin{aligned} \Delta V^* &= 546 - 1.81\,T \\ \Delta V &= -923 + 3.21\,T. \end{aligned} \tag{18}$$

Whether or not the numerical agreement between the coefficients in (17) and (18) is coincidence, it deserves mention.

Once the preceding values for the volume changes are adopted, the remaining constants of equation (10) can be determined. Not only the pH data, but also the activity: temperature and the activity: pressure data of Brown et al. are fitted satisfactorily by equation (19),

$$v = \frac{3.3 \times 10^{23}\, T f_H \times 10^{-\left[\frac{7410}{T} + p\left(\frac{-2.56}{T} + 0.00937\right)\right]}}{1 + \frac{9.0 \times 10^{6}}{(H^+)} \times 10^{-\left[\frac{4120}{T} + p\left(-\frac{4.67}{T} + 0.0171\right)\right]}} \tag{19}$$

where v is measured in micromoles ATP split in one minute by one milligram of myosin, p is measured in atmospheres, and f_H is an undetermined function of pH that is equal to one at pH 8.4. This leads to the following values of the constants in (10):

$$\Delta E^* = 34{,}000 \text{ cal/mole}; \quad \Delta V^* = -483 \pm 1.77 \text{ T in cm}^3\text{/mole}$$
$$\Delta E = 18{,}900 \text{ cal/mole}; \quad \Delta S = 32 \text{ E.U.}; \quad \Delta V = -882 + 3.23 \text{ T in cm}^3\text{/mole.} \qquad (10a)$$

These constants are somewhat different from those of luminescence. The activation energy of 34,000 cal/mole for the rate process (c) is large. The energy of reaction of 18,900 cal/mole for equilibrium (d) is unusually low for a reversible denaturation. The entropy change on denaturation is also relatively small. Entropy, or disorder, increases on denaturation, as expected, but not very much. Most denaturations show an entropy increase of the order of 200 entropy units (Johnson, Eyring and Polissar, '54). This suggests that reversibly denatured myosin still retains a well ordered structure. The numerical values of all these thermodynamic constants are sensitive to the values taken for the temperature dependence of the volume changes and must be regarded not as final figures but as indications of the correct order of magnitude.

In the present paper, reversible denaturation has been regarded as identical with the hydrogen ion dissociation. The inactivated enzyme might, however, exist in two forms, with and without the dissociated hydrogen ion. This would modify equation (7) to read as follows:

$$v = k'_d \frac{M_t}{1 + K'_d\left(1 + \frac{K_h}{(H^+)}\right)} \qquad (7a)$$

where K'_d is the equilibrium for reversible dissociation and K_h the dissociation constant of the acid species of denatured myosin. Either equation will fit the present data equally well. The scatter in the pressure: pH data suggests that there may be pressure-insensitive differences between preparations, which might be referred to differences in K'_d of equation (7a). The scatter may, however, also be caused by sensitivity of the original K_d to factors other than hydrogen ion concentration. The difference in the apparent activation energies of the precipitated and the dialyzed myosins of Brown et al. ('58) may also be referred to one or the other of these possibilities.

SUMMARY

Expressions are derived for the interpretation, on the basis of absolute reaction rate theory, of pressure, temperature and hydrogen ion influences on myosin adenosine triphosphatase activity.

Numerical data that fit the experimental results of Brown and co-workers include the following, which suggest the correct order of magnitude for the thermodynamic values:

(a) in the reversible denaturation of myosin, ΔE is 18,900 cal/mole, ΔS is 32 cal/mole-degree, and ΔV is $(-882 + 3.23\ T)$ cm^3/mole.

(b) for the breakdown of the myosin ATP complex, ΔE^* is 34,000 cal/mole, and ΔV^* is $(-483 + 1.77\ T)$ cm^3/mole.

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EXPERIMENTALLY ALTERED STRUCTURE RELATED TO FUNCTION IN THE LOBSTER AXON WITH AN EXTRAPOLATION TO MOLECULAR MECHANISMS IN EXCITATION

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TWENTY-NINE FIGURES

The relation between macromolecular or colloidal structure and the excitation-conduction-recovery cycle in nerve, and its extension to the phenomena of contraction, secretion, etc. is a central matter in the area of "irritable" structure physiology. This laboratory has used a dual approach to the problem. On the one hand, an attempt has been made to discover structural changes accompanying functional activity, it being hoped that, once isolated, such changes could be manipulated and chemically analyzed (Bryant and Tobias, '55; Shaw and Tobias, '55; Tobias, '52, '52a). In addition however an opposite approach has also been undertaken: Instead of looking for a change of structure with activity, an attempt has been made to produce a known structural change, as by the application of purified enzymes to axons, and to determine its effect on functional activity (Tobias, '55). In the first case activity was examined for detectable, accompanying structural alterations. In the second, experimentally produced structural alteration is being examined for effects it may have on functional activity.

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The latter experiments have shown that either phospholipase A or C applied to the outside of the surviving lobster. giant axon produces functional changes which indicate that the structural integrity of probably the lecithin molecule is essential for normal operation of the nerve machine. Contrariwise, since either trypsin or alpha-chymotrypsin can leave electrical function intact while making the axon very soft, sticky and mechanically fragile, it has tentatively been concluded that none of the substrates of these two enzymes, accessible to them when applied to the axon from outside, is essential for function (Tobias, '55). The word "function" in this report is understood to refer primarily to the phenomena immediately involved in excitation, conduction and recovery.

These observations are important as part of the information allowing further insight into molecular level, structure-function relationships in nerve. Some such information has been available earlier (Bryant and Tobias, '55; Kayushin et al., '54, '55, '56; Kornakova et al., '47, '52; Lyudkovskaya et al., '52; Shaw and Tobias, '55; Tobias et al., '52, '52a, '55), but most of the literature purporting to deal with this problem does not satisfyingly do so, since the experiments usually deal either with structure or function but fail to monitor both at the same time.

To increase understanding of the ultrastructural parameter in the enzyme experiments it seemed that electron microscopy might be useful. The present report deals with such observations of lobster giant axons treated with enzymes and other substances while still alive and submitted to tests of functional competence.

METHODS

The axon preparation, electrophysiological measurements, and the mode of exposure to enzymes have been described earlier (Tobias and Bryant, '55; Tobias, '55). In all cases, the lobster Homarus americanus was used.

Single axon preparation for microscopy has generally been as follows: immerse in cold, fresh, 1% osmium tetroxide in artificial sea water (Prosser, '50) at pH 7.2, and keep in the dark at 3°C for 12–15 minutes with occasional very gentle agitation. All subsequent treatment, up to polymerization of the imbedding matrix, is at room temperature. Wash in distilled water for 30 minutes, changing the water at 15 minutes. Dehydrate for 10–15 minutes in each of a series of alcohols graded 35%, 50%, 70%, 80% and 95%. Dehydrate for 15 minutes in each of three changes of absolute alcohol. Infiltrate for 15 minutes in a solution made of one part absolute alcohol and one part a mixture of methacrylate monomers containing 90% *n*-butyl methacrylate and 10% methyl methacrylate. Infiltrate in pure 9:1 methacrylate mixture for 30 minutes. Infiltrate in the 9:1 methacrylate mixture containing benzoyl peroxide 0.25%, for 30 minutes. Replace with fresh solution and polymerize at 50°C for 24 hours.

For axon bundles the time in solutions were appropriately longer. In some cases it has seemed desirable to use artificial sea water buffered with glycyl glycine for the experiment, and to fix, wash, dehydrate, etc. in each solution for an hour or longer. The latter procedure was followed with the axons of figures 17–23.

Sections were cut with a glass knife on a Porter-Blum microtome and were examined with an RCA electron microscope EMU-2. All illustrations are of axons from the third interganglionic connectives.

While osmium tetroxide is probably not a perfect fixative, it seemed, on the basis of x-ray diffraction experiments, reasonable to use it. Thus, although it is said differentially to intensify and slowly to modify the spacings of the x-ray diffraction bands of frog myelin, the pattern is preserved, and comparison with electronmicrograms suggests that the structure revealed by electron microscopy after OsO_4 treatment "probably resembles very closely the normal structure" (Finean, '54). Even so, one is aware that fixation artifacts almost surely do occur, and it is only to be hoped that internal controls allow them to be taken into account.

At any rate, one test of usefulness of a procedure is whether or not it gives the experimenter new insights into a problem. The following pages suggest that the presently employed techniques satisfy this criterion to a reasonable extent. The interpretations to be discussed do not depend upon any assumption of chemical specificity in the staining.

RESULTS

Immediately fixed whole nerve cords. For these observations whole nerve cord segments were fixed immediately upon excision. This was followed by transection to facilitate penetration, but no further dissection was done. Nor was there prior exposure to any artificial medium. The pictures may be compared with those of Geren and Schmitt ('54).

Figures 1 and 2 illustrate randomly observed axons. They demonstrate the complexly structured, multilaminated, periaxoplasmic layers as well as the semi-regular arrangement and sub-sheath accumulation of so-called mitochondria (compare Geren and Schmitt, '54). Figure 3 shows an inpouching of the just periaxoplasmic layer. It has been suggested that such herniations may represent mitochondrial entry into the axoplasm (Geren and Schmitt, '54). Figure 4 shows such bodies just inside and just outside the axoplasmic surface. No compelling evidence that they cross the interface has been seen in these experiments, though figures 3, 4, 6 and 7 are suggestive.

Doubling of the densely osmiophilic layer at the axoplasmic surface, of the mitochondrial surface layer and of layers in the sheath is clearly seen in figures 3, 5, 6 and 7. In addition, figure 6 shows irregular interruptions in the axoplasmic surface layer. It is as if the axoplasmic surface structure is, at this level of resolution, in some regions a continuous sandwich of two dense osmiophilic layers separated by a less dense interval, and in other regions is made of discontinuous sandwich segments with closed edges which, in cross section, appear as circlets and variably elongated ellipses. One is tempted to speculate about an axonal surface mosaic, but the present experiments do not warrant this, since such interruptions have been seen in only a few instances.

Figure 8 shows that a simpler interaxonic structure can also be found, though in these experiments this is the sole instance when the writer has seen such a region composed of only two osmiophilic lamellae between two axons. There is no compelling reason to believe that this is a functional junction, a synapse, though the possibility does occur to one.

Figures 9 and 10 show segments of a giant axon fixed in the intact nerve cord. Some of the surface lamellae have been tipped sideways and allow a view through them. They are not completely homogeneous, but neither do they exhibit any regular structure. The axoplasm is sparsely populated by nodulated rodlets (see also fig. 17, 18, 19, 21, 22, 23). From darkfield observations the lobster axoplasm is known to be much more fluid than that of the squid, and in the living state has been described as containing chains of particles preferentially oriented along the axonal long axis (Tobias and Bryant, '55).

Experimental results. To see the experimentally produced changes dealt with in this report one will evaluate electronmicrograms of axons all of which were isolated by dissection in a salt water medium. This seems a strenuous experience at least so far as structure is concerned, and it will immediately be apparent that even the control axons, simply isolated and exposed to the medium which supports function for hours (figs. 11, 12, 13, 14), already appear to be very different from the immediately fixed, unmanipulated ones which have never been exposed to any artificial physiological medium (figs. 1–10). Thus, one will compare experimentally modified axons with baseline axons, themselves already much changed. In all cases, subtle details of structure have been obscured or destroyed by exposure to the experimental solution. Therefore these data are not to be viewed as contributing to the electron microscopy of well preserved normal structure. It has simply turned out that osmium and electron microscopy have yielded, even under these disadvantageous conditions, some little information which can be correlated with the functional studies. From such a point of view, even the large changes already seen in the soaking controls have intrinsic interest; the examples show that apparently considerable change, at least so far as it is expressed by reaction with osmium tetroxide, can be tolerated without loss of electrical function. What the strict morphologist might dismiss with the words "post mortem change" becomes, when viewed in the light of concomitant functional status, an interesting phenomenon.

Controls. Soaking controls were obtained simultaneously with a number of the experiments and their appearance can be variable. Figure 11 shows an axon fixed after about 6 minutes exposure to sea water, the time required for isolation. Figures 12, 13 and 14 show axons kept isolated in sea water for about three hours. In all cases there was a decrease in density combined with swelling and spreading of the surface layers. It is entirely likely that both water uptake and removal of soluble components underlie this change in appearance, and are probably to be related to the drift in scattered light intensity exhibited by resting nerve (Bryant and Tobias, '55; Shaw and Tobias, '55). Higher magnification yielded no more information. All of these axons conducted normally just before fixation.

Protease treatment. Many of the isolated axons, whether only soaked or otherwise experimentally treated, when finally visualized had somehow lost their axoplasm. Fortunately it was retained in enough so that one could tentatively evaluate the state of the axoplasmic-extraaxoplasmic interface.

Figures 15 and 16 show two axons which were exposed to a mixture of trypsin and alpha chymotrypsin, 1 mg of each per cm^3 of sea water, for two and three hours respectively. Figures 17, 18, 19 and 20 show low power views, and figures 21, 22 and 23 show high magnification views of several regions of another axon so treated which retained its axoplasm. There is gross granular destruction and deformation of all the extraaxoplasmic structures extending, in most though not all regions, all the way in to the axoplasmic surface layer, and in many places even this most internal layer seems to have been changed. Thus, figure 21 shows such a boundary layer where a considerable span of the ribbon-like surface film remains intact though at some points it appears rarefied; mechanical breaks seem fairly easily detectable. Figure 22 shows a region which appears changed all the way in to the axoplasm. Figure 23 shows two semi-parallel pieces of surface layers which, though still recognizable, seem greatly modified and eroded.

These axons, and others of similar appearance, all conducted normally just before immersion in the OsO_4 solution.

Several points merit special discussion and interpretation, but these can best be considered after examining the results of other experiments.

Phospholipase A. Phospholipase A, which splits lecithin into a fatty acid and lysolecithin, was obtained by using Naia Naia venom (Tobias, '55). The enzyme is functionally isolatable by controlled heating at low pH. Unheated venom, venom heated so as to preserve the phospholipase and venom heated so as to destroy it were all used. Such axons are rendered inexcitable by the phospholipase A (Tobias, '55) but they have not so far exhibited any convincing structural changes which can with assurance be attributed to the phospholipase A treatment. The sort of fragmentation seen after trypsin plus chymotrypsin has not been seen. Nor are there changes comparable to those found after exposure to phospholipase C (see below).

Phospholipase C. Fractionated *Cl. welchii* toxin, as used earlier (Tobias, '55), was the source of phospholipase C. This enzyme splits phosphorylcholine off of lecithin leaving a diglyceride residue.

Figures 24 and 25 show axons treated with this material for thirty minutes. Both became inexcitable before fixation. Such treatment produces a distinctive appearance. Laminae are still present and recognizable, but are widely spread, stretched looking and tortuous. Dense black particles are randomly distributed among them. It was reported earlier (Tobias, '55) that such axons appear, even with the dissecting microscope, to be cloudy and granular, as if frosted or superficially powdered with many small, light-scattering particles, and it was assumed at the time that these particles represent loci of phospholipid residue coalescence after phospholipase hydrolysis. This seems to have been a reasonable guess, and the dense osmiophilic particles seen electronmicroscopically are, in all likelihood, such osmium stained, coalesced, diglyceride residues.

The axoplasmic-extraaxoplasmic interface of such axons is widely disrupted. In addition, the inset, figure 26, shows that mitochondrial surfaces were modified by the enzyme, this also indicating penetration into the axoplasm. The dense mitochondrial surface layer disappears, and small black particles, again suggesting lipid coalescence, appear in its stead. This is further evidence that the mitochondrial surface contains phospholipid (see also Nygaard et al., '54).

Figure 27 is of special interest both for its own sake and as a control. The axons of figures 24 and 25 were treated with a phospholipase C-rich preparation contaminated slightly with collagenase. It was earlier shown that the phospholipase C and not the collagenase was responsible for functional failure (Tobias, '55), but now a similar discrimination seemed desirable for correlation with the structural changes. Figure 27 represents one of several axons treated with a collagenase rich, phospholipase poor preparation. The duration and condition of exposure were identical with those for the axons of figures 24 and 25.

This axon, and the others so treated, showed beginning functional changes by the time of fixation, but they were still conducting. The usual sequence following phospholipase C treatment is rising threshold, slowing conduction velocity, broadening and failing action potential, and, finally inexcitability followed by depolarization. The axon shown here, figure 27, had just begun to show rising threshold and slowing conduction. For correlation with these beginning functional changes it is seen that structural changes were limited to relatively peripheral elements. The more central laminae are not stretched or tortouous looking, and the dense osmiophilic particles are peripheral.

Clearly the structural changes, as also the functional ones were due primarily to the phospholipase.

Digitonin. Digitonin, 0.1 mM, in sea water, rapidly produced inexcitability. Figure 28 shows an electronmicrograph of such an axon. Digitonin treated axons resemble those exposed to phospholipase C in that the laminae, though present, are widely spread, stretched and tortuous. Dense osmiophilic particles again are present but now in a more finely divided state. With the dissecting microscope these axons appeared to be somewhat cloudy, but not powdered or granular as were the phsopholipase C-treated ones. The small particles here could be in part cholesterol digitonide.

DISCUSSION

1. To discuss first the experiments with trypsin and alpha chymotrypsin: These enzymes disrupt a variety of protein linkages. When applied to the axon they make it soft, sticky and fragile though leaving electrical function intact. This refers to exposures up to about three hours long. Carried on long enough such treatment might, of course, destroy the axon functionally. The observations show that such treatment alters the extraaxoplasmic lamellae, and two findings suggest that some sort of change may extend all the way to the axoplasmic surface: (a) figure 7 shows no detectable difference between the innermost, axoplasmically applied lamella and more peripheral ones. If the enzymes can alter the outer layers then there is no a priori reason on the basis of such findings to exclude the possibility that they can do the same to the inner one. (b) Figures 18, 19, 20, 22 and 23 strongly suggest that the innermost layer has somehow been modified by this treatment.

This matter is, however, in no sense regarded as settled, and further evidence is needed concerning how far such particles may penetrate without disrupting function. First of all, as noted earlier, the large background of damage due simply to soaking the axons makes it difficult to resolve and evaluate any detailed changes possibly due to the experimental procedure; only the grosser changes can be relied upon. Secondly, Northrop ('26) showed long ago that amoebae are killed by injected proteases which they tolerate very well when applied to the surface. Obviously however the matter is of interest, since the depth to which particles of molecular weight 15,100 (trypsin) to 21,600 (alpha chymotrypsin) (Edsall, '53) could penetrate without disrupting function would be important in evaluating cell surface structure. (See note added in press.)

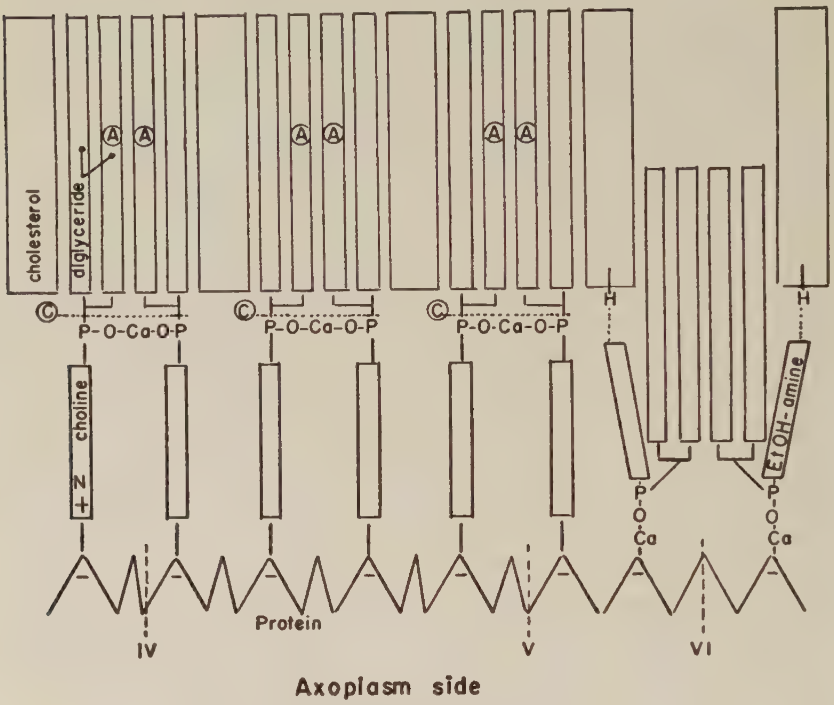
2. Either of the phospholipases, A or C, produces rising threshold, slowing conduction, broadening and gradual decrease of the spike followed by inexcitability and depolarization. Phospholipase A has produced no clearly characteristic structural change so far convincingly demonstrable either by light microscopy or by electron microscopy. Phospholipase C, on the other hand, does produce characteristic structural changes. Light microscopy of the unfixed, surviving axon shows a gradual accumulation of surface distributed, light scattering particles; electron microscopy demonstrates densely osmiophilic particles, irregular in size and shape, and more or less randomly distributed among the surface lamellae. These presumably are droplets of coalesced diglyceride resulting from enzymatic hydrolysis of phospholipid. In contrast to the protease treated axons, the lamellae are still present and recognizable though displaced from each other and tortuous.

The beginning changes in threshold and conduction velocity which occur even when only the outer lamellae are disrupted with phospholipase C show that all of these layers play some role in the signals conduction process. Tentatively one might guess that as the number of intact layers is decreased the transsurface capacity may increase and account for rising threshold and decreasing conduction velocity.

3. An attempt to understand the above results is made easier by reference to a diagram of a hypothetical structure

which we will consider may exist at the cell surface (text fig. 1). It will be assumed the surface lamellae are constructed roughly as shown, out of radially oriented phospholipid and cholesterol, and tangentially oriented protein with calcium in between, and that the proposed structure also holds for the layer most intimately applied to the axoplasm, the so-called axolemma, or, in the emphasis of many physiologists, "*The* membrane." Approximating from data of Brante ('49) on lecithin, cephalin and cholesterol, and from Salach ('57) on Ca, there is, on a molar basis, in lobster nerve, about 5 times as much lecithin as cephalin, about half as much cholesterol as lecithin plus cephalin, and about 20% more equivalents of positive charges available from Ca than needed to couple the lecithins as shown. The calcium remaining after such lecithin coupling might bind some cephalin cholesterol duos, hydrogen bonded between the cholesterol OH and the ethanolamine NH_2, to the protein substratum. If this were uniformly the case then there would not be enough calcium to go around, but some phospholipid could well be retained in the structure by surface forces alone. At any rate, the precise stoichiometry is not to be taken too seriously at this time; the point is simply that for lobster nerve there is about enough of the materials in question to go around if related to each other as suggested by the diagram. The positively charged ammonium nitrogen end of choline could serve as a means of associating the lecithin with negatively charged sites on the underlying protein skeleton. Some remaining cholesterol could interact directly with peptide linkage sites in the protein providing an additional means of associating the protein skeleton with the lipid layer (not shown); similarly some phospholipid oxygen could couple directly to positively charged protein sites (not shown). Evidence supporting such possibilities is derived from a number of sources (Winkler and Bungenberg de Jong, '41; Schmitt, Bear and Palmer, '41; Frey-Wyssling, '48; Booij, '49; Dervichian, '49; Doty and Schulman, '49; Matalon and Schulman, '49; Finean, '53; Eley and Hedge, '56).

It should be kept in mind that, to the extent that such a schema is accurate, the proposed structure will be a dynamic one, the picture representing possibilities which are realized for some of the time, i.e., probabilities.



Text figure 1

Now consider the enzyme actions in the context of this diagram: The proteases cleave the protein skeleton at such sites as IV, V and VI, for instance, though how they penetrate the lipid barriers is not clear. So long as the axon is not mechanically disturbed the elements remain tenuously held in place by virtue of the hydrophobic character of the attached lipid layer and the bindings between it and the protein underlay, and both resting and action potentials are maintained. Because of the protein cleavages, however, the axon is mechanically more fragile than normally (Tobias, '55), and when assaulted by the osmium solution, water, alcohols, etc., it goes to pieces and appears finally as a fragmented ring of granular debris.

Phospholipase A cleaves the fatty acid A free from its glycerol, and this functionally inactivates the axon. It is no longer able to support either resting or action potentials, and, it is anticipated, normal ion segregation will disappear. However no convincing structural changes have yet been seen in this case. Perhaps the cleaved fatty acid does not leave the cell surface matrix, as suggested for red cells treated in a similar though not identical fashion (Ballantine and Parpart, '40). If this is the case then spatial relations are maintained because all the elements remain roughly in their proper positions, and fragmentation is avoided because the protein skeleton remains intact.

Phospholipase C cleaves the entire diglyceride from its phosphoryl choline moiety, as at sites labelled C. This inactivates the axon functionally, outwardly in the same fashion as does phospholipase A. Now, however, the diglyceride residues, and perhaps also the loosely held cholesterol, leave the film and coalesce to form the particles seen both by light and electron microscopy. Again the protein structure persists. But contrary to the situation following A position hydrolysis, in this case the orienting forces due to association with the phospholipid layer no longer exist and the protein becomes spatially disarranged and distorted, though it remains more or less in one piece.

The results strongly imply that functional integrity is more dependent upon precise lipid organization than upon precise protein organization. In addition, it would appear that the spatial arrangement of the protein is to a large degree dependent upon the integrity of its lipid companion. One is tempted to extrapolate therefore, that if changes in such a structural complex underlie and permit the ion movements characteristic of activity, then such events are more likely to be produced by changes induced in a key lipid element than in a protein one alone. Furthermore, it is suggested that the sites in such a structural complex most sensitive to change by current, which in cells is ionic current and which can therefore change ion concentrations only at regions of phase discontinuity, would be in the region of the interface between lipid and protein where are located the positive choline-nitrogen link to protein and the negative phosphate-oxygen links to calcium.

Considering the phenomenon of excitation in this context; during the period of triggering by the spread of catelectrotonus, K^+ ions will be moved from the axoplasm into this interfacial region where they will decrease the probability, and therefore the time, of association of N^+ with the negative protein site and of Ca^{++} with the negative oxygen sites. Similarly, any associations of phospholipid-oxygen directly with positively charged protein sites, or of phospholipid-oxygen, through Ca^{++}, to negatively charged protein sites would be diminished. In addition, the K^+ ions driven to the N^+-linked-to-protein site will indirectly release Ca^{++} from the oxygens, because the now free N^+ will tend to form an internally neutralized zwitterion with its own phosphate group oxygen. Also, conceivably by releasing acetylcholine from its bound precursor, the K^+ may make available a second N^+ in the acetylcholine for competition with the phospholipid N^+. At any rate, the point is that K^+ driven into the interface region by the long axis voltage gradient which exists between a now-active and an about-to-become-active region, i.e., by catelectrotonus, can by a variety of means which will call for no chemical specificity, affect electrostatic bonds there in such a way as to favor spatial dispersion or separation of structural elements, and this is suggested as the first step in permitting the secondary, passive and grosser movements of Na^+ and K^+ which are the action current.

Release of the bonds to protein might well allow protein fragments to enter the axoplasm where they could contribute to changes in light scattering (Bryant and Tobias, '55), and to an increase in osmotic pressure resulting in dimensional changes (Hill, '50; Bryant and Tobias, '55). Such protein released with each impulse would have, however, to be replaced, and, since protein adsorbed beneath a lipid film tends to be denatured (Eley and Hedge, '56), with each impulse additional denatured protein would be released to the axoplasm there to be refurbished by metabolism. It is interesting that recent data suggest an increase in reversibly denatured protein with activity (Ungar et al., '57).

Nothing has been said about equating such a structure with the concept of a unique, single, metastable membrane at the axoplasmic surface whose state is thought by many to define, or indeed to be synonymous with, whether the axon is resting or active. The evidence bearing on this matter is discussed elsewhere in detail (Tobias and Nelson, '57). Suffice it here to say that neither electron microscopy nor any other technique has demonstrated that an innermost, axoplasmically applied layer exists which is chemically or structurally different from all other layers outside it (see for example fig. 7). However, it is clear that the innermost layer is unique in one way, i.e., it is the only one in contact with axoplasm on one side and non-axoplasm on the other. Since many properties of an interface are determined by the composition of the bulk phases which contact it, it may well be that this innermost layer will have unique properties of biological importance. For example the lipid element (text fig. 1) will control the orientation of the immediately subjacent protein layer, and this, acting as a template may determine the structure and qualitative properties of the superficial axoplasm.

4. If the suggested structure really exists then perhaps removing phospholipid and cholesterol with digitonin (McEwen and MacLachlin, '41; Fieser and Fieser, '44) would have consequences similar to those following treatment with phospholipase C. Treatment with digitonin did result in rapid onset of inexcitability, and, as seen from text figure 1, the appearance of such axons is reminiscent of those treated with phospholipase C. The fact that the osmiophilic particles are smaller than after phospholipase C treatment may be due

to the fact that they were formed more rapidly. The digitonin produced inexcitability almost immediately, indicating rapid penetration; the phospholipase C doses had been chosen to require 15–30 minutes to produce inexcitability.

5. On similar grounds it might be expected that calcium removal would result in some sort of change in lamellar organization. It has been reported for squid fibers that Ca depletion produces lamellar spreading (Geren and Schmitt, '54) though details are lacking.

In concluding, one may point out that the schema is potentially testable in a further way. Thus after treatment with phospholipase C it may be possible physically to separate the cleaved diglyceride from the tissue mass, and, if solubility conditions are favorable, chemical analysis should show that both C and choline either will remain preferentially with the protein fraction or will be indiscriminately distributed. On the other hand, treatment with phospholipase D (not yet tried) which cleaves choline from the phosphoric acid group might cause more Ca to come away with the lipid fraction.

SUMMARY AND CONCLUSIONS

1. The electron microscopically demonstrable structure of lobster axons has been illustrated.

2. The functional and certain structural consequences of exposing lobster giant axons to sea water, to trypsin plus alpha chymotrypsin, to phospholipase A or C, or to digitonin have been investigated and illustrated. Difficulties in interpreting certain of the apparent structural changes are appreciable because of the background alteration produced by control procedures. Only the grosser effects have been relied upon.

3. It is concluded that the structural integrity of axonal phospholipid is obligatory if excitability and impulse conduction are to persist; on the other hand, apparently a degree of disruption of surface protein can be tolerated; however, how slight or how great a change in protein is compatible

with normal excitability is not known, and such experiments as these probably cannot answer this question.

4. A hypothetical structural complex consisting of lecithin, cephalin, cholesterol, protein and calcium is used in an attempt to explain the observed results: The experimental findings are compatible with this structure, and certain other experiments are suggested by it.

5. It is postulated that if changes in such a structural complex underlie and permit the ion movements of activity, then such events are more likely to depend upon changes in a lipid element than in a protein one alone, and that, furthermore, the sensitive sites in such a structural complex, as regards change by ionic current, will be in the region of the negatively charged oxygen in phospholipid phosphate and positively charged nitrogen in phospholipid choline.

6. A further hypothesis concerning molecular structural events during the early period of triggering of excitation has been sketched.

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ADDENDUM

Note added in press: Subsequent experiments with squid axons support the conclusion that trypsin, at least, can penetrate into the axoplasm without disrupting function.

Abbreviations to plates

Ax, axoplasm; SL, surface lamellae.

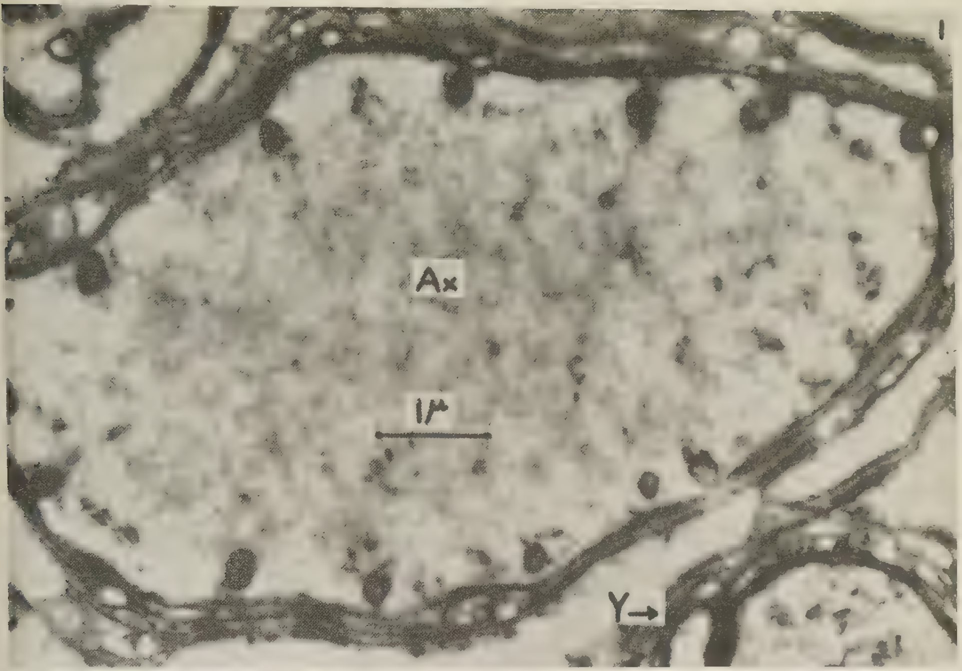
PLATE 1

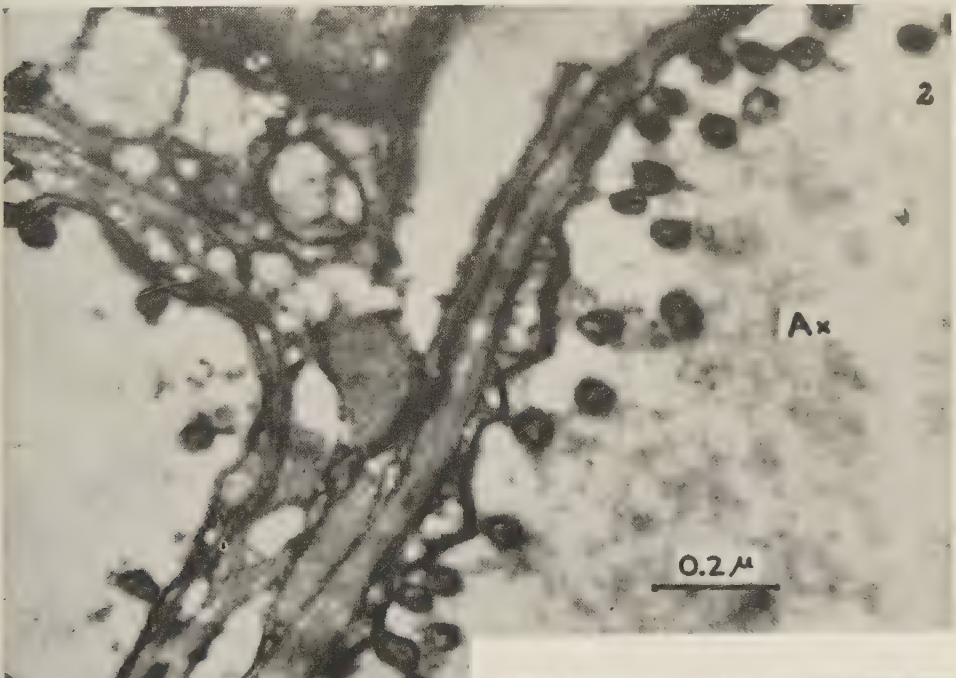
EXPLANATION OF FIGURES

1 Axon from intact nerve cord. ''Y'' shows branching of sheath layer.

2 Axon from intact nerve cord.

3 Invagination of axoplasmic surface layer.


1
Ax
1μ
Y→


2
Ax
0.2 μ

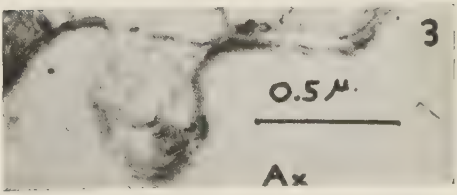

3
0.5 μ
Ax

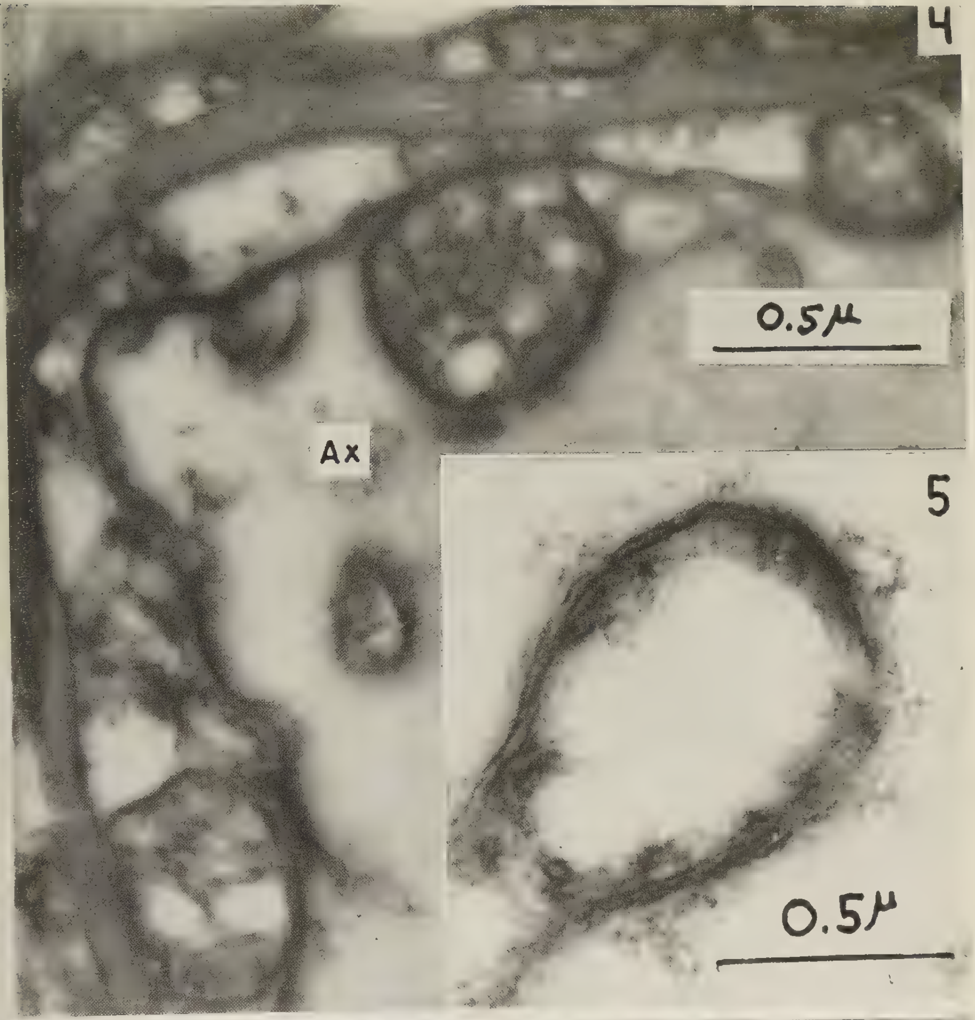
PLATE 2

EXPLANATION OF FIGURES

4 Note ''mitochondria'' just inside and just outside axoplasmic surface.

5 Note doubling of ''mitochondrial'' surface layer.

6 Note apparent interruptions, at arrows, of axoplasm surface layer.


4
0.5μ
Ax
5
0.5μ

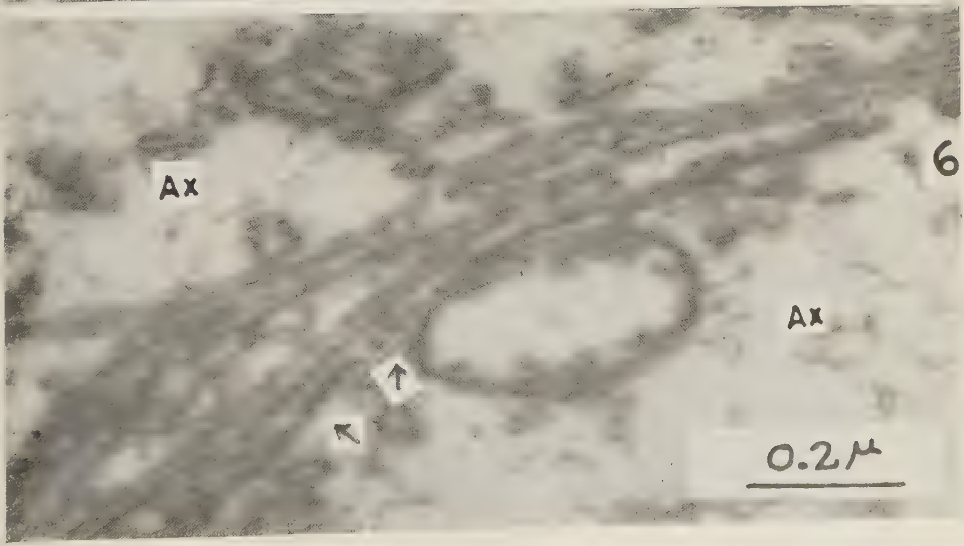

6
Ax
Ax
0.2μ

PLATE 3

EXPLANATION OF FIGURES

7 Note similarity of innermost double layer to those farther out.

8 Shows a region where two axons are separated by only one double layer.

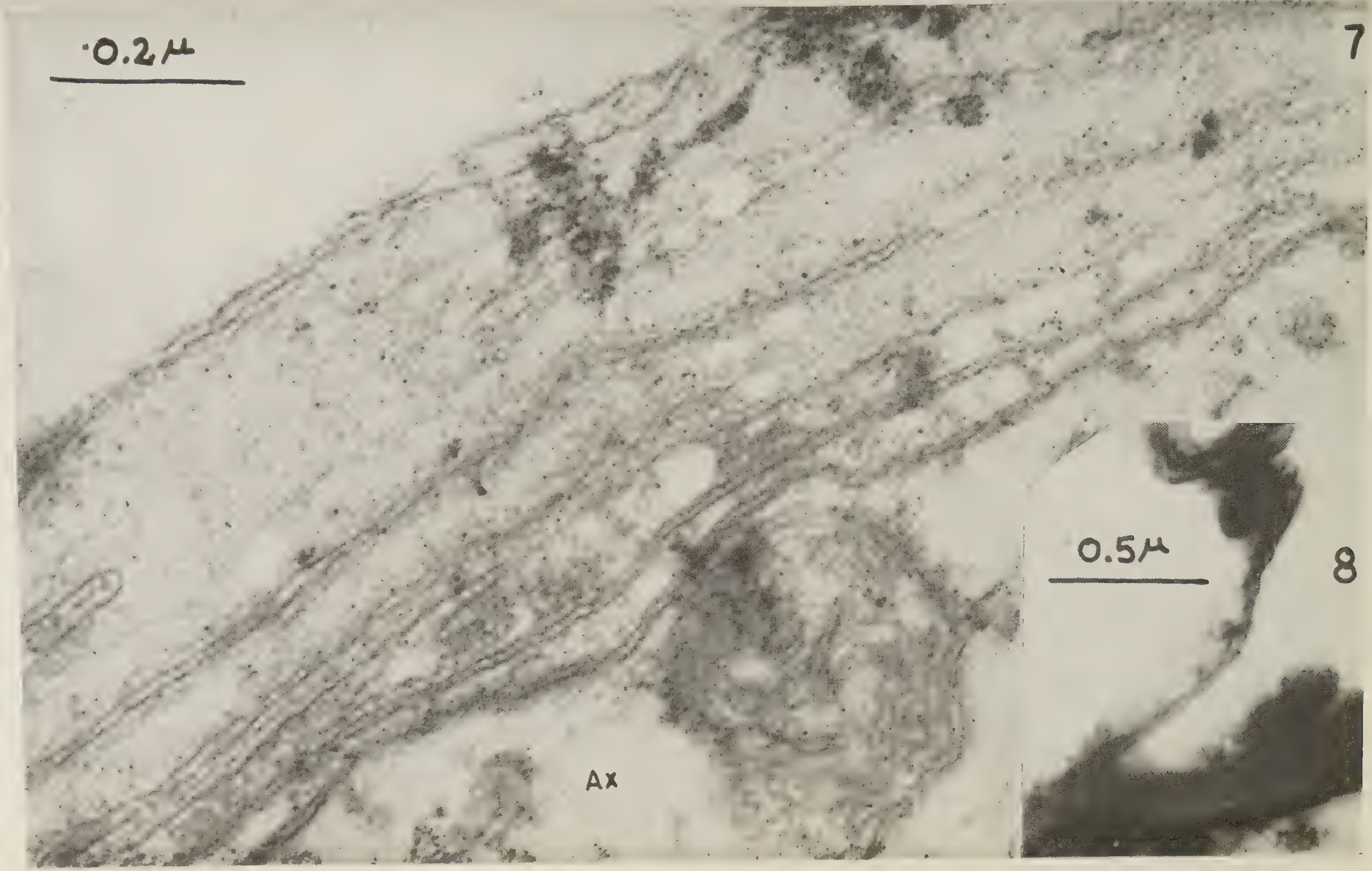

0.2μ
7
0.5μ
8
Ax

PLATE 4

EXPLANATION OF FIGURES

9–10 Note appearance of surface lamellae tipped sideways and viewed as ribbons.

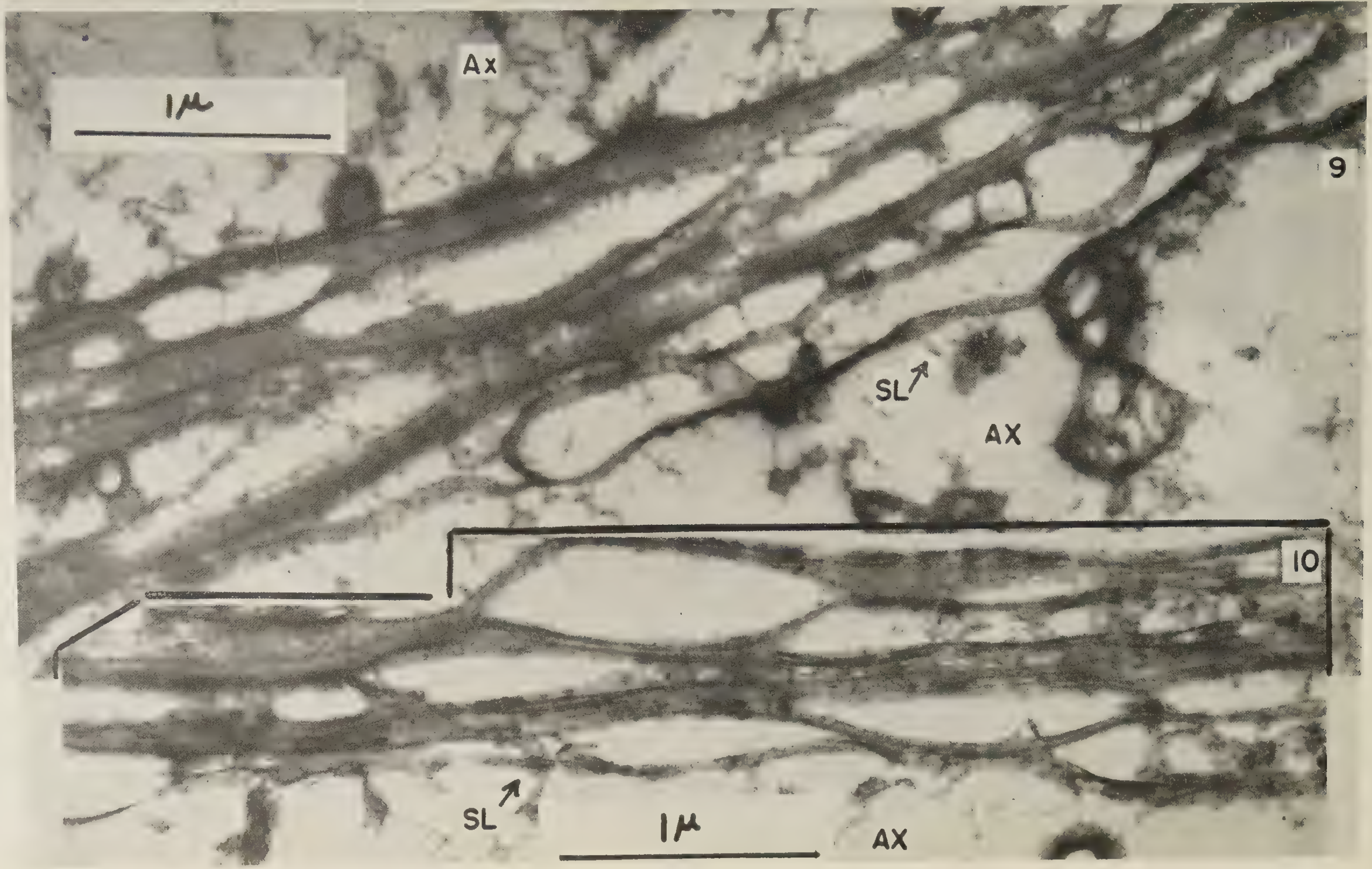
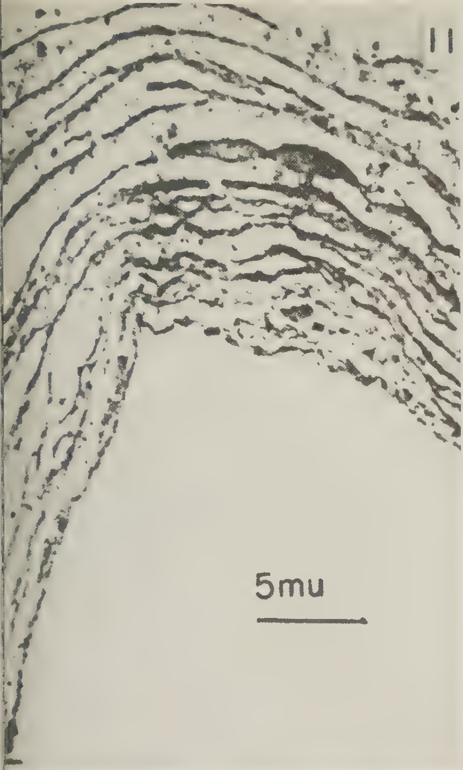
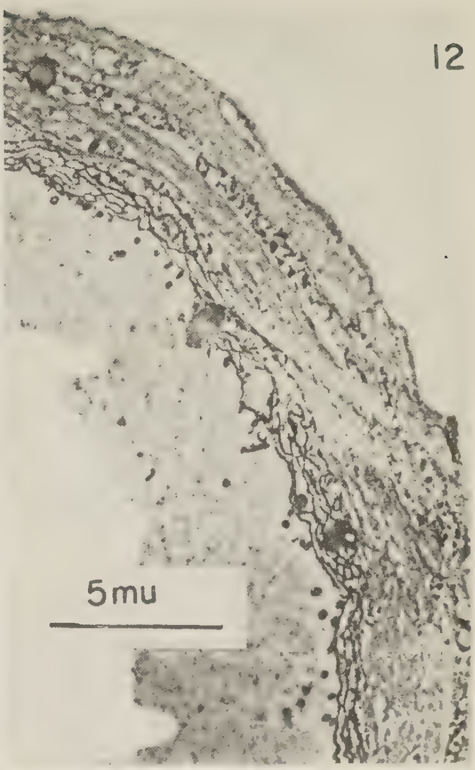

Ax
1μ
9
SL
AX
10
SL
1μ
AX

PLATE 5

EXPLANATION OF FIGURES

11–14 Isolated giant axons soaked in sea water for 0.1, 3.25, 3 and 3 hours respectively. All conducting normally at time of fixation.


11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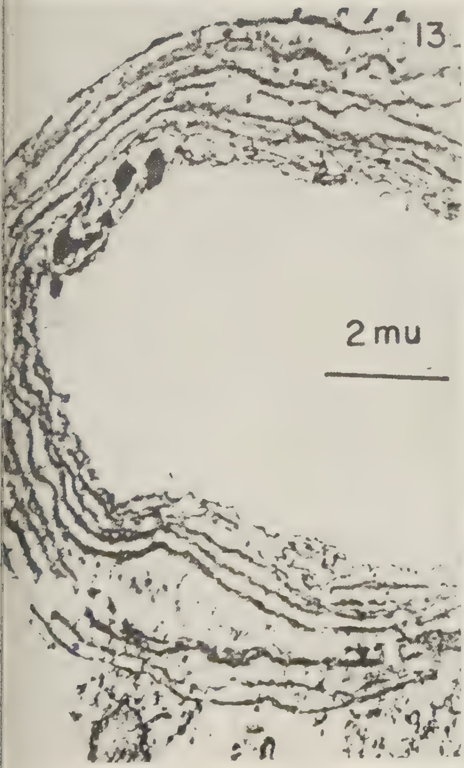

13

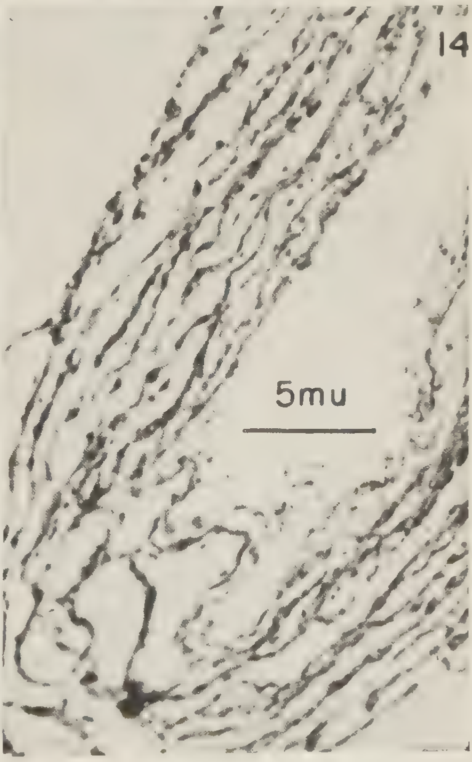
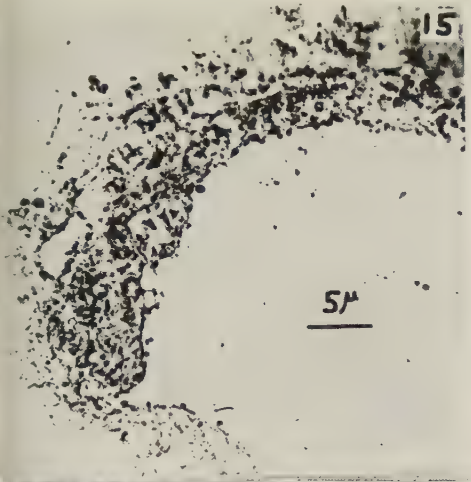
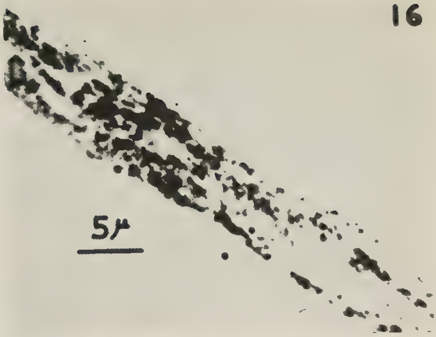

14

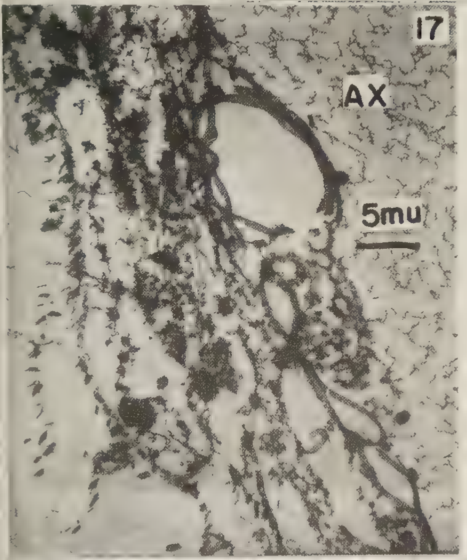
PLATE 6

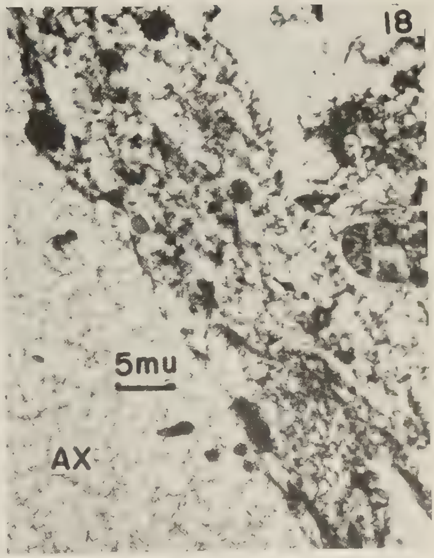
EXPLANATION OF FIGURES

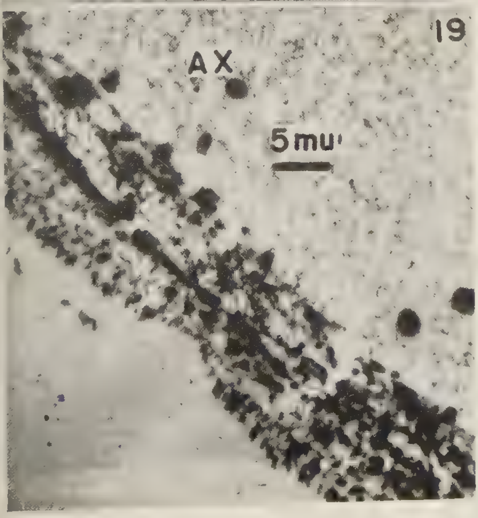
15–20 Views of axons treated with a mixture of trypsin and alpha chymotrypsin, 1 mg of each per cm^3 of sea water. Figure 15 and 16 are each of a different axon. Figures 17–20 are 4 views of a third axon at different points along the long axis. All exposed to enzyme medium for about three hours at room temperature. All conducting normally at time of fixation.


15
5μ


16
5μ


17
AX
5mu


18
5mu
AX


19
AX
5mu

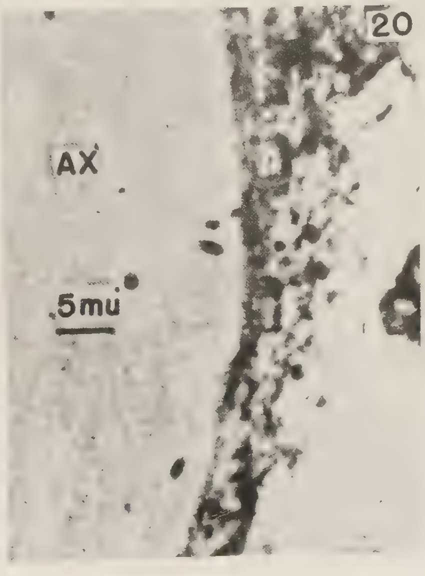

20
AX
5mu

PLATE 7

EXPLANATION OF FIGURES

21–23 Higher power views of axon shown in figures 17–20.

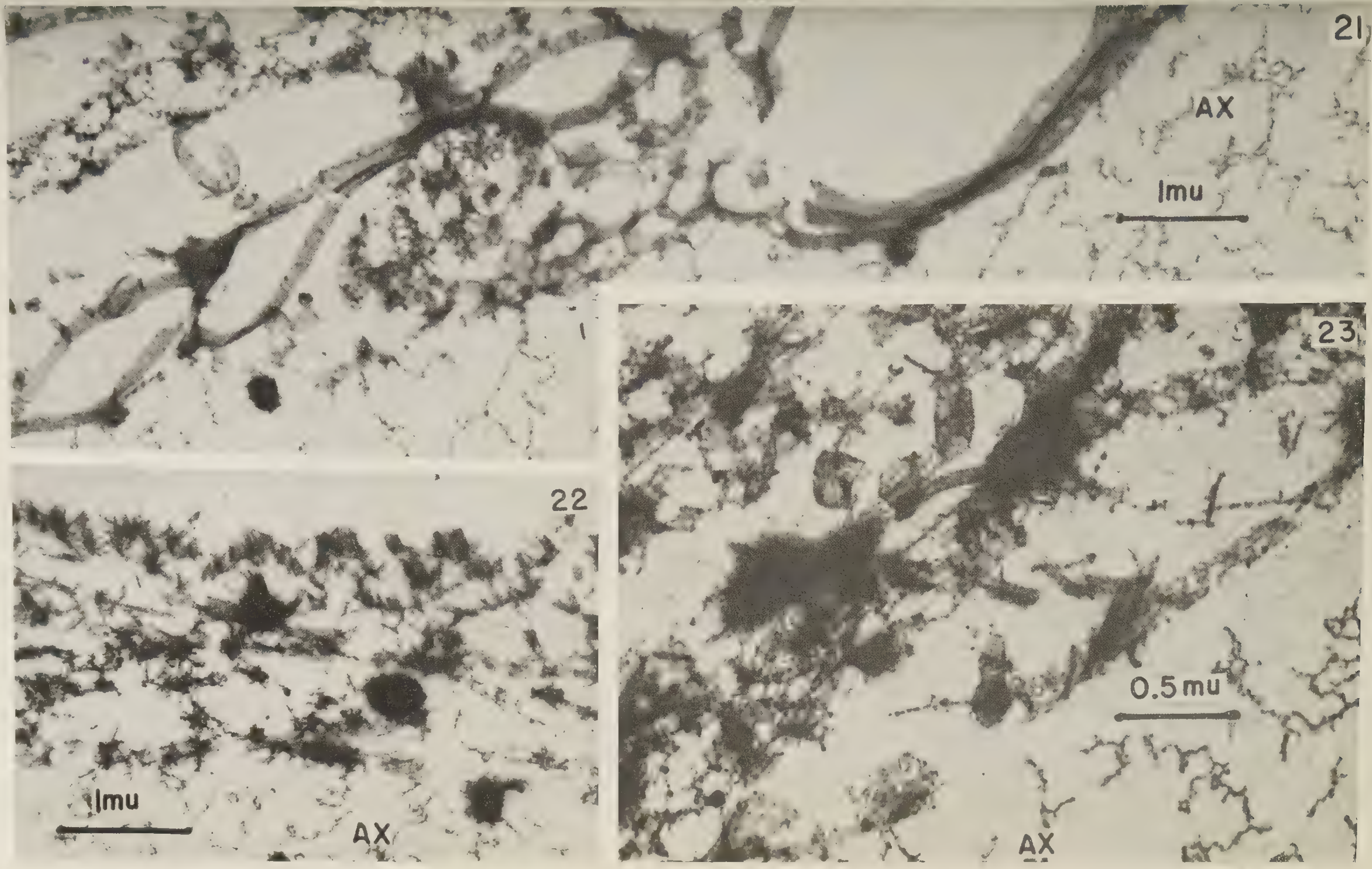
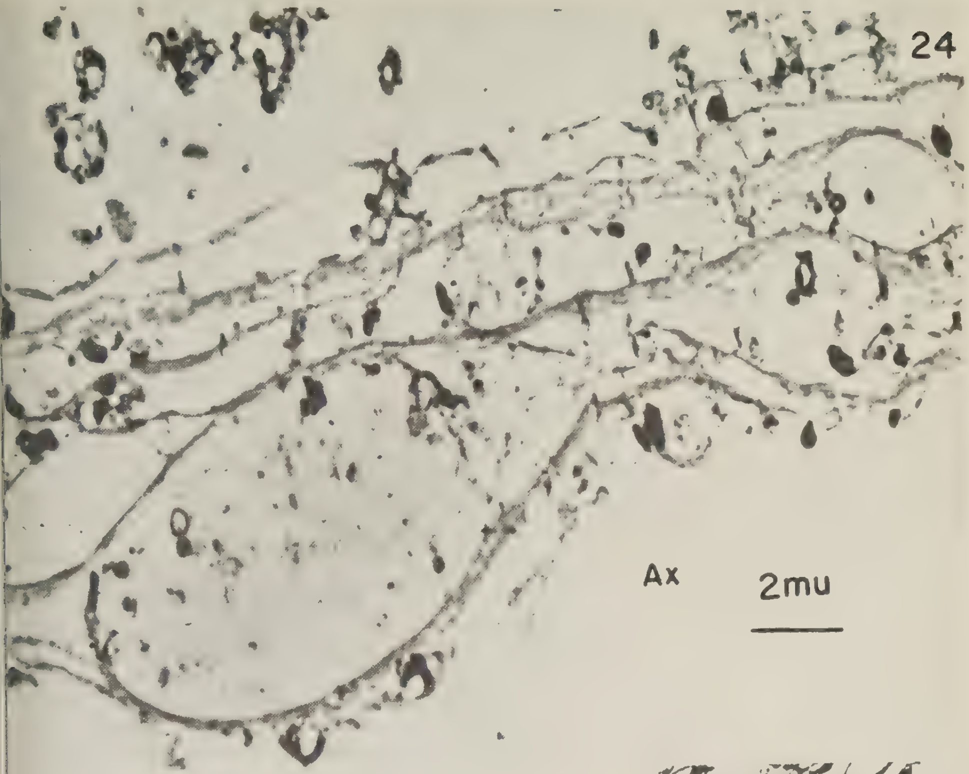

21
AX
1mu
23
0.5mu
AX
22
1mu
AX

PLATE 8

EXPLANATION OF FIGURES

24 25 Axons treated with phospholipase C-rich, collagenase-poor fraction of *cl. welchii* toxin in sea water for 30 min. Appeared as if powdered with many small light-scattering particles when examined with dissecting microscope at 36 ×, before fixation. Inexcitable at time of fixation.

26 Two ''mitochondria'' from phospholipase C-treated axon. Note loss of surface layer on one; the other looks as if surface material had coalesced mainly at one spot to give a signet ring type of appearance. See also in axoplasm of figure 25.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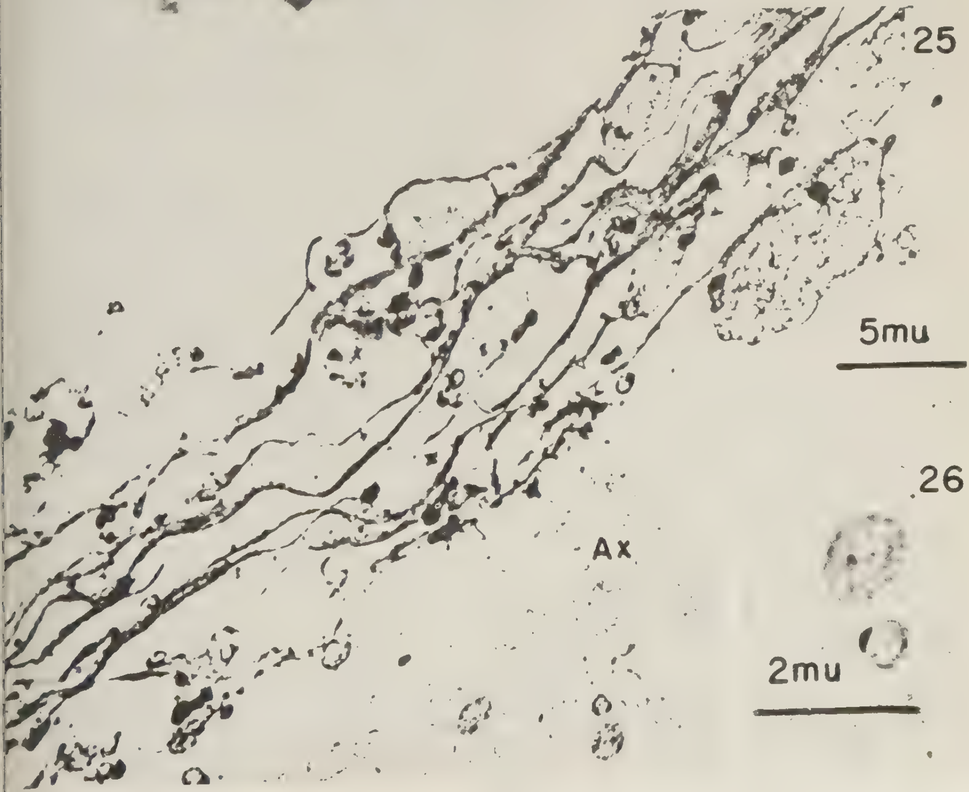
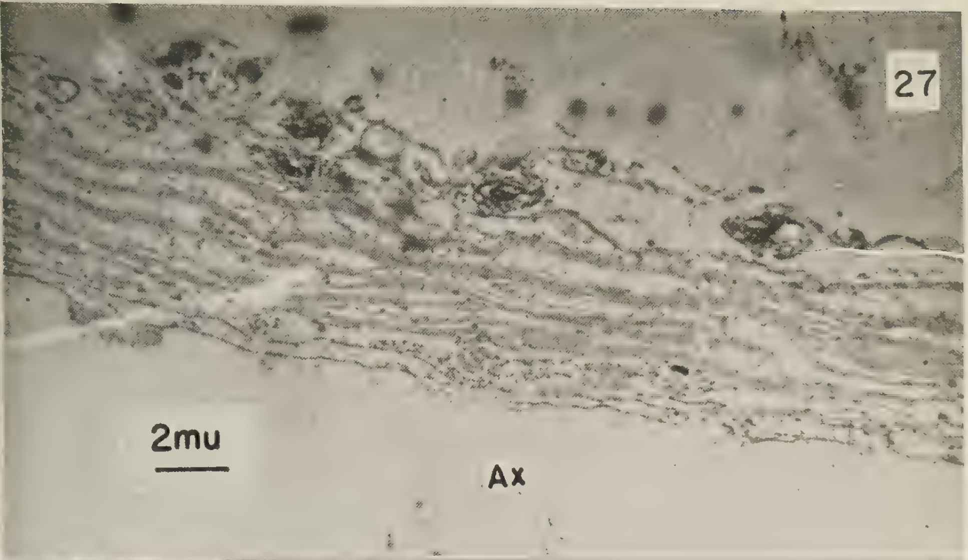

25
26

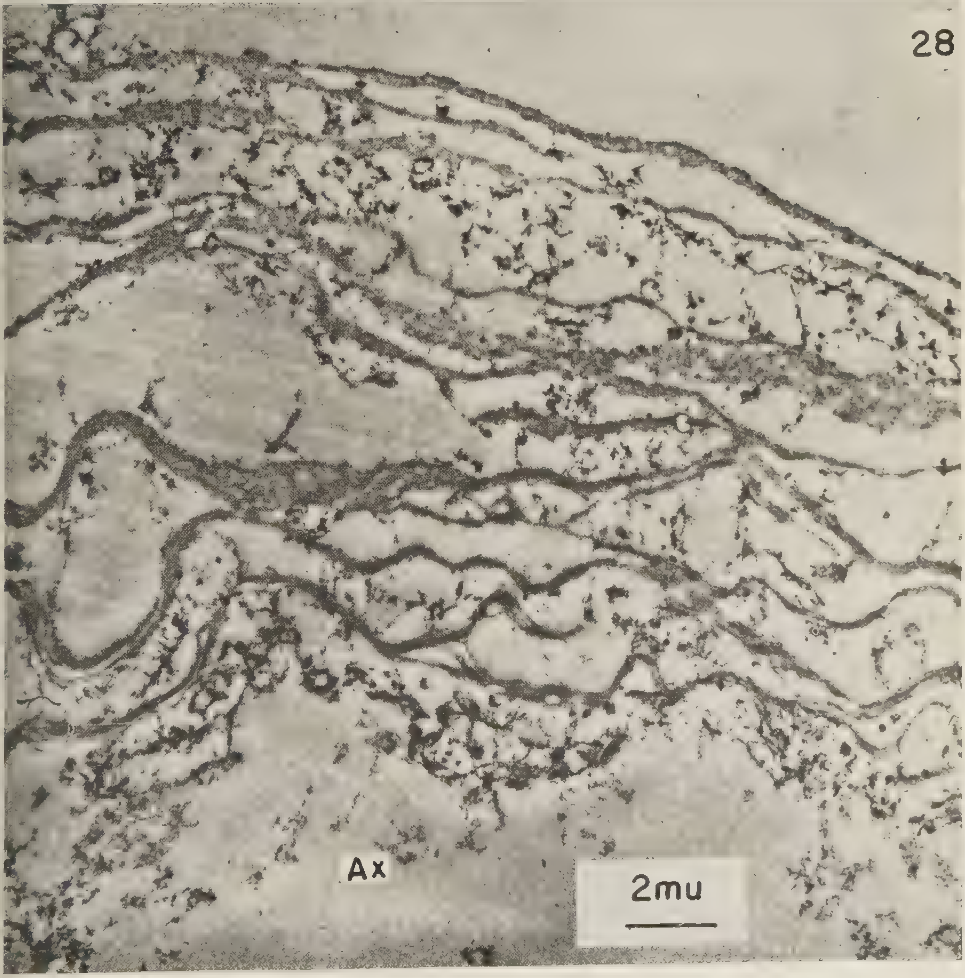
PLATE 9

EXPLANATION OF FIGURES

27 Axon treated with phospholipase-poor, collagenase-rich fraction of *cl. welchii* toxin in sea water for 30 min. Still conducting at time of fixation, but had begun to show rising threshold and slowing conduction velocity. Note that changes are limited to outer parts of axon.

28 Axon treated with digitonin in sea water, 0.1 mM, for three hrs. Inexcitable at time of fixation.


27
2mu
Ax


28
Ax
2mu

EFFECTS OF CERTAIN ENZYMES ON NODE OF RANVIER EXCITABILITY WITH OBSERVATIONS ON SUBMICROSCOPIC STRUCTURE[1]

PHILLIP G. NELSON[2]
Department of Physiology, University of Chicago

SIX FIGURES

A number of schemata have been presented for the molecular anatomy of cell surface structure. Most of these involve some sort of combination of protein with lipid, especially phospholipid. Ponder ('48) and Ballentine and Parpart ('52) have emphasized the possibility of a mosaic structure, with foci of radially oriented lipid molecules in a stroma of protein. Schmidt ('33), Schmitt et al. ('39, '41) and Finean ('53, '53a) have obtained x-ray evidence that the myelin of nerve consists of lamellae composed of radially oriented lipid molecules bounded by tangentially oriented protein. Winkler and Bungenberg de Jong ('41) and Booij ('49, '54), working with *in vitro* systems of protein and lipid, have formulated many of the patterns of interaction of these compounds and the effects that various ions have on such interaction, leading to a "complex" theory for biologic membranes in which protein, cations and lipid are bound together.

Efforts have been made to get at the role that these constituents may play in cell function. Parpart and Ballentine

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[2] National Science Foundation Post-Doctoral Fellow. This work has been submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Physiology, in the University of Chicago.

('40) treated erythrocytes with trypsin or with lipase. The lipase strongly affected permeability but trypsin had no measurable effect. Papain and mexicain, proteolytic enzymes, are said to have little effect on red cell permeability, although they do have some hemolytic activity (quoted by Ponder, '48). Chymotrypsin was shown to be hemolytic only after red cells were exposed to it for many hours (Tobias, '55). Sarkar and Maitra ('50) tested the effect of cobra venom, which contains phospholipase A as well as many other enzymes, on neuromuscular function. In frog nerve-muscle preparations, venom applied either to the neuromuscular junctional region or to the muscle directly prevented function. When the venom was applied to the nerve alone, however, function of the nerve-muscle preparation was unimpaired. An integrated study of structure-function relationships in unmyelinated nerve has been carried out by Tobias ('55, '58). Using enzymes as molecular dissecting tools, he studied the functional results of exposing axons to ribonuclease, hyaluronidase, collagenase, proteases and phospholipases. Only treatment with phospholipolytic enzymes proved to destroy function in the lobster giant axon. Electron microscopy of protease treated fibers showed structural disorganization. Phospholipase A treatment produced changes difficult to characterize if any; phospholipase C treatment produced a characteristic structural alteration.

This technique of more or less selective enzymatic alteration of structure as a means toward understanding the molecular basis of excitability has been applied to myelinated nerve in the work to be described here. These experiments, analagous to those done on unmyelinated fibers, were initially planned for several reasons: first, it was thought that the excitable structure at the node might be more accessible to the action of externally applied large molecules such as enzymes than it is in the unmyelinated fibers. Several layers of myelin-like material surround the entire so-called unmyelinated axon, while at the node in myelinated fibers it has been thought that the myelin may be completely interrupted (Hess and Lansing, '52). Second, because of the findings with unmyelinated axons (Tobias, '55), it seemed important to check, with isolated myelinated fibers, the negative findings that Sarkar and Maitra ('50) reported for cobra venom applied to whole frog nerve. Third, since the active excitatory process in myelinated nerve occurs only at the node, it was hoped that some structural correlates of this functional specialization would be found. As it has turned out, some information has been obtained concerning the structure of the node and the effects of several enzymes on nodal excitability have been observed. No clear-cut information has been obtained yet as to the effects of the enzymes on nodal structure.

METHODS

Single fibers were isolated from small branches of the sciatic nerve near the knee in grass frogs. The nerve bundle was then transected at 1 — 1.5 cm from the node on each side and the fiber was mounted on a bridge insulator (after Tasaki, '51). Stimulation and recording were done in conventional fashion with platinum electrodes. The entire preparation was kept in a damp box shielded with aluminum foil, at room temperature. Solutions were changed with a hand pipette.

The following enzymes were used: (1) proteases, a combination of Armour salt-free trypsin and alpha chymotrypsin, each in a concentration of 1 mg per cm.^{3} These enzymes attack a number of peptide and amino bonds (Bergmann and Fruton, '41; Neurath and Schwert, '50), and also have some esterase activity (Schwert et al., '48). Inactivation for control purposes was achieved with diisopropylfluorophosphate (Jansen et al., '49). (2) Phospholipase A, from cobra venom, functionally isolated by heating at 100°C and at pH 5.5 for 15 minutes. This procedure destroys all enzymes known to be present except the phospholipase A (Braganca and Quastel, '52, '53). A control, with all enzymatic activity, including that of phospholipase A, destroyed, was prepared by heating the venom at 100°C at pH 8.5 for 60 minutes. This enzyme splits lecithin, yielding the alpha fatty acid and lysolecithin; it also releases choline esterase from erythrocytes (Grieg and Gibbons, '55), frees acetylcholine from the bound state in brain tissue (Gautrelet and Corteggiani, '39; Braganca and Quastel, '52), and decreases the succinoxidase activity of mitochondria (Nygaard and Sumner, '52; Nygaard et al., '54). (3) Phospholipase C from ultrafiltrates of *Cl. welchii* cultures; several different fractions have been used, as will be noted. This enzyme splits lecithin into a diglyceride and phosphorylcholine. It attacks sphingomelin to some extent (Macfarlane, '48) and inhibits Mg activated adenosinetriphosphatase (Kielly and Myerhof, '50).

Assay of protease activity by the haemoglobin technique of Anson ('38) showed the enzymes to remain active for lengths of time comparable to those of the nerve experiments (see also Muller and Yamanouchi, '57). Hemolytic techniques (Roth and Pillemer, '53) were used for phospholipase A and C assay.

For electron microscopy, the following procedure was used. Small branches of the frog sciatic nerve were dissected, desheathed and teased apart. Some were then quickly fixed while others were first soaked or treated with enzyme solutions identically as those used in the functional studies. Fixation and staining was done by immersion for 15 minutes in a cold, acetate-veronal buffered 1% osmium tetroxide solution made isotonic with a balanced salt solution. Dehydration in increasingly concentrated ethanol, over a three to four hour period, and impregnation with 10:90 methyl: butyl methacrylate was followed by isolation of single fibers and subsequent imbedding and polymerization at 54°C for about 24 hours. It was then possible to trim down to the individual node and make longitudinal sections through it. Some sections were also made from whole nerve. Formvar films and an RCA EMU-2 electron microscope were used.

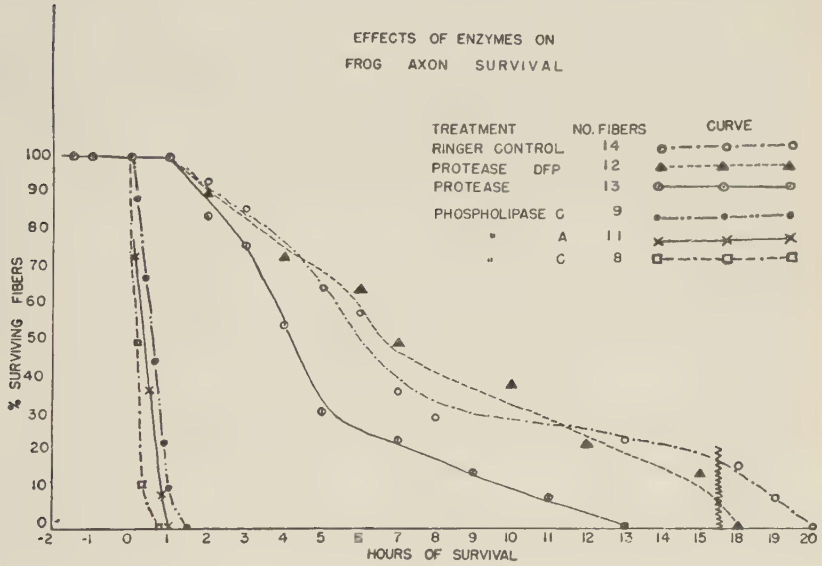
RESULTS

Nodal structure. The electron micrographs to be discussed have been selected not because they show any one feature perfectly, but because they show, at least in part, all the important features seen in the experiments. Therefore, they represent a compromise and should be so evaluated.

The structure of the normal node may be described by listing the structures which one encounters on progressing from the outside of the fiber inward to the nodal axoplasm. Sections from quickly fixed whole nerve demonstrate the features to be enumerated (figs. 1 and 5). First there is an osmiophilic, outer limiting membrane (OLM) which continues laterally from the nodal region to lie over an amorphous, rather thick (up to 0.1 μ) layer of low electron density, which seems to be Schwann cell cytoplasm (SC) on the surface of the myelin sheath. In figure 1 there appears to be a discontinuity in this layer where a body from the perinodal space penetrates it. The perinodal space contains many of these bodies (PNB), oval or ribbon-like in form, and having some internal structure. At the nodal axoplasmic surface there is a second densely osmiophilic layer (ASL) continuous across the node and morphologically indistinguishable from similar layers in the myelin sheath. It is 65–70 Å thick and has, in these experiments, revealed no internal substructure. It differs from the more peripheral lamellae in that it remains in close contact with the axoplasm in areas of the internodium where the (other) myelin is elevated. As the node is approached along the long axis of the axon the myelin lamellae are seen to peel off (fig. 4) and turn in to terminate, in many cases if not all, in bulbous enlargements (MLT) some of which may contain granules, and which are in more or less close apposition to the axoplasmic surface layer described above. Just beneath the nodal axoplasmic surface a cluster of vesicle like bodies (SSV), about 200-600 Å in diameter, may be seen in some preparations (fig. 5). These are smaller than the perinodal space bodies and are reminiscent of the presynaptic vesicles seen at central nervous system synapses (DeRobertis, '56; DeRobertis and Franchi, '56; Palay, '56). The axoplasmic structure is primarily filamentous or fibrillar, but more centrally located vesicles or annulae are sometimes seen (fig.

3). Other features of normal nodal structure are shown in figures 2, 3 and 4. These pictures are of a fiber treated with proteases for two hours. However, all the features to be noted have also been seen in control fibers. No definite statement as to the effect of proteases on nodal structure can yet be made except for the rather negative one that after two hours of protease action the structures that are demonstrated still remain relatively intact. In figure 2, the complete interruption of the myelin sheath at the node is apparent. The myelin sheath is connected to the axoplasmic surface by many tendril-like groups of lamellae, which as noted above, are seen in figures 3 and 4 to be continuous with the myelin lamellae themselves. Extra-axoplasmic, perinodal bodies occur at E in figure 2 and, at the other side of the axoplasm, vesicle like bodies (SSV) are again seen just below the surface. The axoplasmic surface layer in figures 2 and 3 is thicker than in figure 1 and seems to be irregularly discontinuous. This appearance has also been seen in untreated control fibers and so cannot be ascribed to the proteolytic treatment. In figure 2 large axoplasmic bodies, mitochondria (?), occur near the node, but these are also seen in the internodal axoplasm.

Effects of enzymes on excitability. Having a description of the nodal area, one may now consider the functional consequences of treating this region with proteases or phospholipases. The data are summarized in text figure 1 which shows the percentage of surviving fibers as a function of time. Ability of the node to produce an action potential when electrically stimulated was the criterion of survival. It can be seen from the figure and from table 1, that there was a slight difference, of borderline significance, between the survival of the protease and control fibers, as if the proteases slightly accelerated deterioration. There was, by contrast, a highly significant difference between all the phospholipase treated fibers and the controls. These latter enzymes rapidly produced inexcitability. It should be pointed out that lysolecithin, a product of phospholipase A hydrolysis of lecithin, is toxic for frog nerve. However, phosphoryl choline and diglyceride, the products of phospholipase C activity, are both non-toxic (Tobias, '55). The *Cl. welchii* material which contained the phospholipase C was contaminated by two other potentially active compounds. These were a so-called theta toxin which is hemolytic in the presence of reducing agents but whose substrate is unknown, and a collagenase. It was therefore necessary to determine whether the effect of the preparation on nerve function was due to the action of these contaminants.



Text figure 1

To do this, the effect of a control preparation with low phospholipase C activity but containing large amounts of theta toxin and collagenase was tested. It failed to block any of 8 axons in two hours (not shown). This is to be compared with curve 4 which shows the prompt loss of excitability produced by a preparation containing less theta toxin and collagenase than the control, but with more phospholipase C activity. Another preparation, containing very little theta toxin and collagenase, but with a high phospholipase C content,

TABLE 1

Time required for enzymes to produce nodal inexcitability

CURVE NO.	TREATMENT OF AXONS	NO. OF AXONS	AVER. SURVIVAL, HOURS (MEAN ± S. E.)	TIME REQUIRED TO INACTIVATE 50% OF FIBERS, HOURS	LONGEST SURVIVAL, HOURS
1	Controls in Ringer	14	8.4 ± 6.2	6.7	20.2
2	DFP inactivated proteases	12	8.8 ± 5.3	7.6	17.4
3	Active proteases	13	5.1 ± 3.4	4.2	12.5
4	Phospholipase C (low theta toxin and low collagenase)	9	0.6 ± 0.4	0.6	1.25
5	Phospholipase A	11	0.5 ± 0.3	0.5	0.9
6	Phospholipase C (high theta toxin and high collagenase)	8	0.2 ± 0.2	0.4	0.75

Statistical evaluation:

Difference between protease treatment and Ringer control; $P = < 0.1$

Difference between protease treatment and DFP protease control; $P = < 0.05$

Difference between all phospholipase treatment and Ringer control; $P = < 0.01$

blocked three axons in less than an hour (not shown). It is clear therefore that phospholipase C itself can produce loss of excitability. Curve 6 shows that high phospholipase C concentration combined with high theta toxin and collagenase may have produced the most rapid block of all: The difference is not statistically significant as shown, but suggests that experiments should probably be done to determine whether collagenase or theta toxin may potentiate the action of phospholipase C.

The peak action current of control nodes varied between 0.4 and 2.0×10^{-9} amp. This usually remained relatively constant for several hours and then slowly declined, with a decrease in rise rate, over a period of an hour or more to a new stable value. Protease treated fibers showed qualitatively similar changes but with a slightly accelerated deterioration of the action current corresponding to the slightly earlier onset of inexcitability. A greatly accelerated deterioration with no greater change in spike slope than shown by the controls followed phospholipase treatment.

DISCUSSION

The fact, as stated before, that the axoplasmic surface layer at the node is structurally similar to the osmiophilic layers of the myelin sheath is susceptible of several interpretations. Thus, this layer may have the same structure and composition as the outer myelin lamellae, and differ from them only in being continuous across the node instead of terminating there. Or, the surface layer may be chemically as well as developmentally unique. If the theory of their origin from Schwann cell membrane is correct (Geren, '45), then a similarity between the myelin lamellae and some other cell membrane as the axolemma would not be surprising. This matter and evidence bearing on it are discussed elsewhere (Tobias and Nelson, '58). In figure 3 an axoplasmic surface layer structurally distinguishable from the myelin lamellae is seen, but this is in an axon which has been experimentally treated and the result therefore is still inconclusive. The results are consistent with the view that the Schwann cell membrane and cytoplasm are continuous across the node. Any statement about the nature of the intra- and extranodal vesicles and bodies would be purely speculative. (See note added in press.)

From the functional studies it is concluded that enzymatic degradation of phospholipid in myelinated nerve results in loss of excitability. How such phospholipolysis leads to inexcitability, however, is not clear. This matter has been discussed elsewhere (Tobias, '55); thus, it may be that phospholipid molecules, e.g., lecithin, are an essential part of the metastable, excitable structure, and destruction of this phospholipid therefore may result directly in loss of excitability. Perhaps local acetylcholine release (Braganca and Quastel, '52) or adenosinetriphosphatase inactivation (Kielly and Myerhof, '50) are responsible. Perhaps destruction of lipid in the myelin sheath so reduces its insulating properties that the flow of action current occurs mainly across the myelin sheath rather than selectively through the nodal area, thereby making current in the external recording circuit indetectably small. Similarly, myelinolysis might allow leakage of stimulating current across the sheath, so that the nodal current would become subthreshold even for high stimulus voltages. This latter mechanism seems somewhat unlikely however because of the occasional observation that when the node in the lateral pick-up pool was not cocainized, its activity persisted for some time after the adjacent, enzyme treated node became inexcitable. If leakage of stimulus current were to account for failure of excitation then this lateral pool also should not fire.

The effect of the protease on excitability, if real, is a late and gradual one. That the integrity of the accessible protein substrates of these enzymes is essential for excitation is therefore unlikely. It is more likely that the protease effect is a nonspecific one, as, for example, a decrease in mechanical strength of the fiber. The possibility must be borne in mind, as is also the case in the experiments with lobster axons (Tobias, '58) that the protease may not produce a marked effect simply because it may not penetrate to the region where its crucial substrate is located.

SUMMARY AND CONCLUSIONS

1. Disruption of phospholipid by phospholipases applied to the nodal region of isolated, myelinated frog axons results in loss of excitability. This is consistent with earlier findings on unmyelinated nerve, and implicates phospholipids in the excitability process.

2. Treatment with proteases results in much later functional changes, if any, of gradual onset. The difference in survival times of control and protease treated fibers is of low statistical significance.

3. Some structural features of the node of Ranvier, as revealed by electron microscopy, have been presented.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the aid and encouragement of Dr. Julian M. Tobias. Dr. Hewson Swift kindly gave instruction and aid in the parts of this work involving electron microscopy. Dr. Albert Zeller, of Northwestern University, gave advice concerning some of the enzymes used, and supplied the phospholipase A preparation. Drs. Pillemer and Roth of Western Reserve University furnished the phospholipase C preparation. The Merck Company of West Point, Pennsylvania, supplied the diisopropylfluorophosphate.

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ADDENDUM

Note added in press: The beautiful work of Uzman and Nogueira-Graf (J. Biophys. Biochem. Cytol., *3:* 589 (1957) on nodal ultrastructure appeared after this paper had gone to press.

ABBREVIATIONS FOR PLATES

M, Myelin
ASL, Axoplasmic surface layer
AX, Axoplasm
MLT, Myelin lamellar termini
PNS, Perinodal space
PNB, Perinodal space bodies
SSV, Nodal subsurface vesicles
MIT, Mitochondria
SC, Schwann cell cytoplasm
OLM, Outer limiting membrane

PLATE 1

EXPLANATION OF FIGURE

1 × 100,000. Quickly fixed whole nerve. This is a longitudinal section through the nodal region, as shown in the inset. A distinct osmiophilic layer 65–70 Å thick is continuous across the node at the axoplasmic surface. Amorphous material, external to the myelin sheath, and presumably Schwann cell cytoplasm, extends into the perinodal space. Bodies with some internal structure occur in the perinodal space, and one may be seen to penetrate the Schwann cell layer. The myelin lammellae terminate, in part at least, in bulbous endings which are closely applied to the axoplasmic surface.

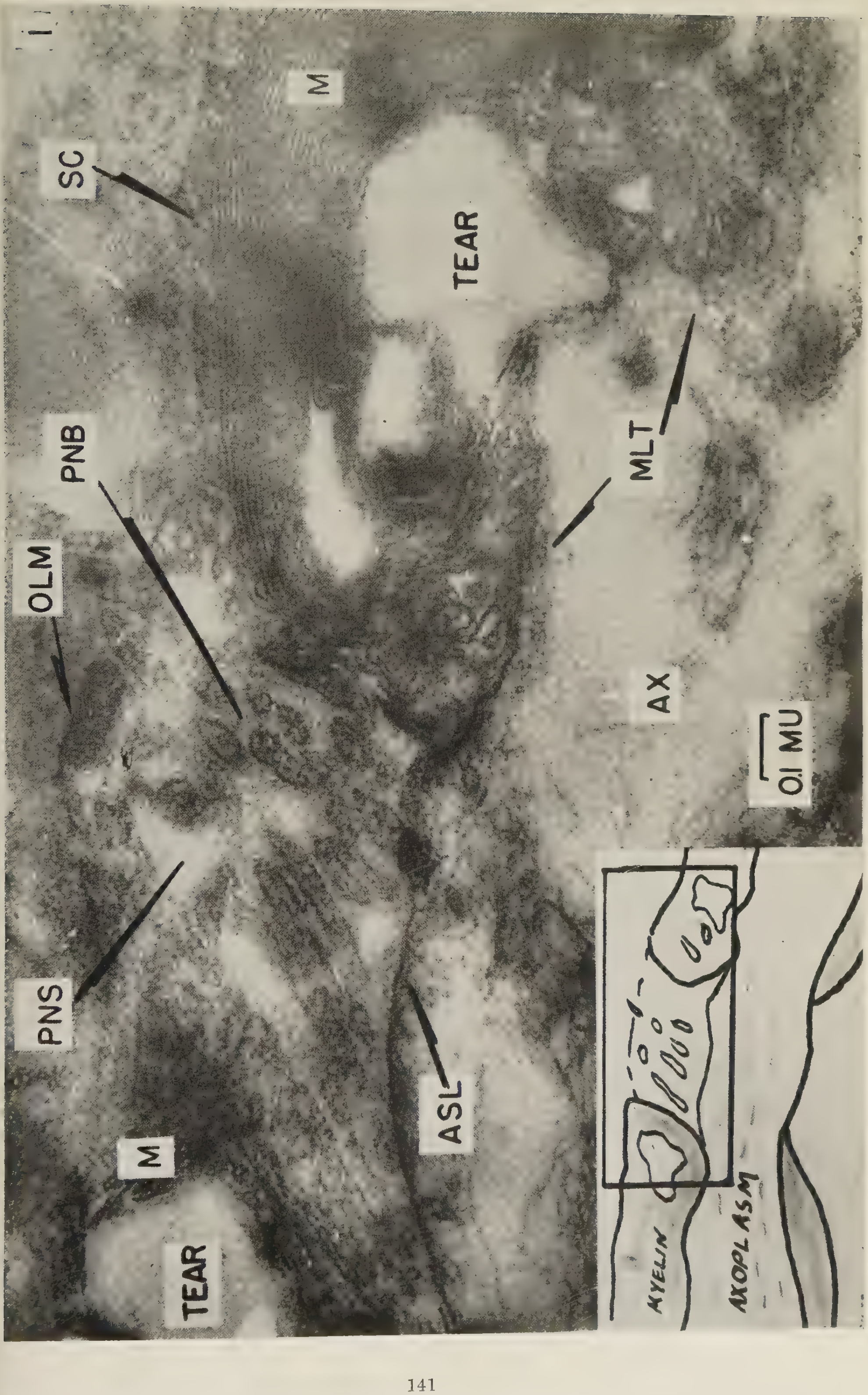
1
SC
M
TEAR
PNB
OLM
MLT
AX
0.1 MU
PNS
ASL
M
TEAR
MYELIN
AXOPLASM

PLATE 2

EXPLANATION OF FIGURE

2 × 10,000. Single fiber soaked two hours in protease solution (1 mg per cm^3 each of trypsin and chymotrypsin). The myelin sheath has been lifted away from the axon except in the nodal region. Numerous tendril-like lamellae connect the myelin sheath with the axon near the node. The myelin is interrupted at the node. Extra-axonal bodies occur near the node at E, and at the other side of the node small vesicle-like intra-axonal bodies are seen just below the nodal surface. Several mitochondria occur near the node.

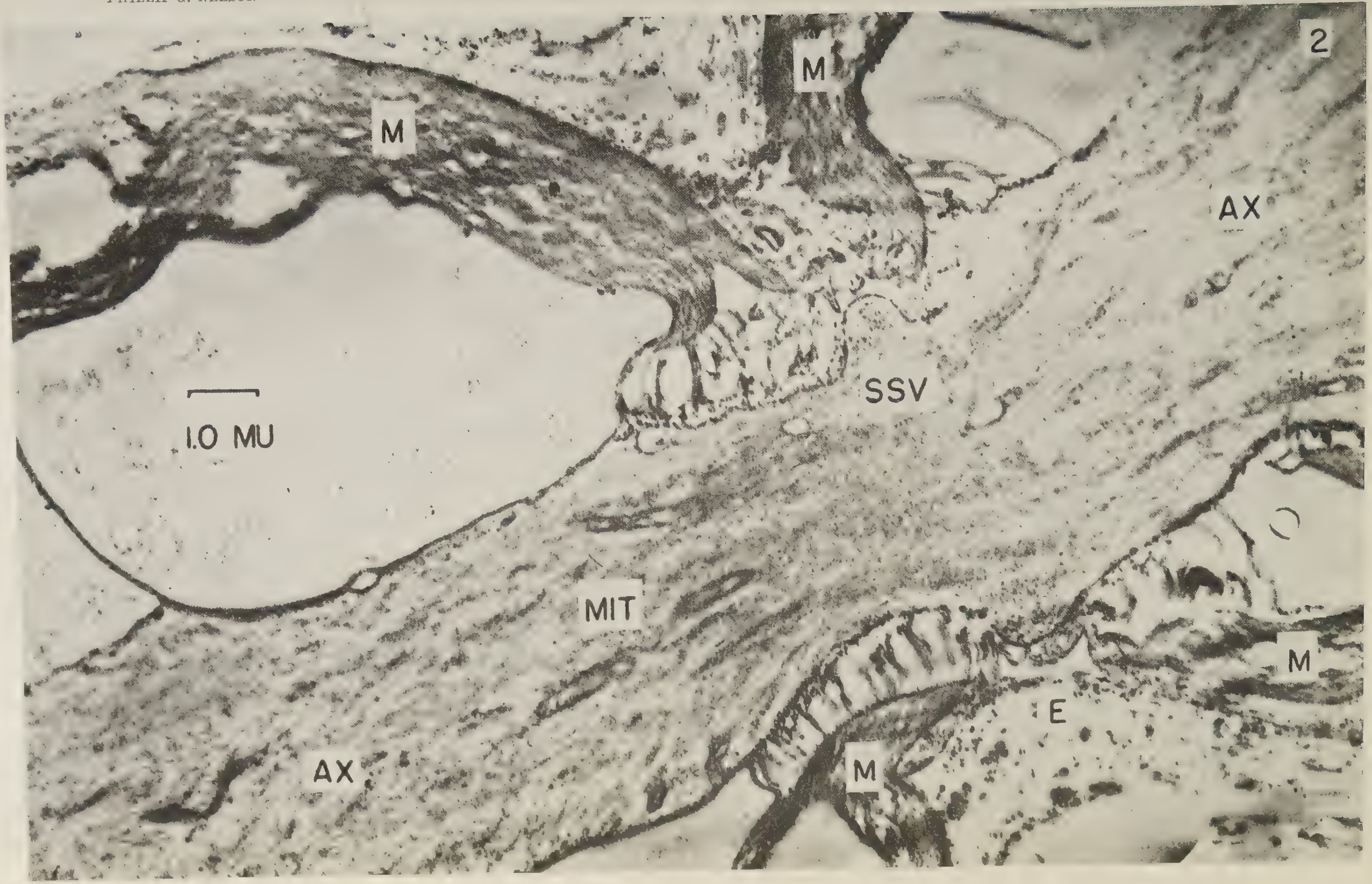
M
2
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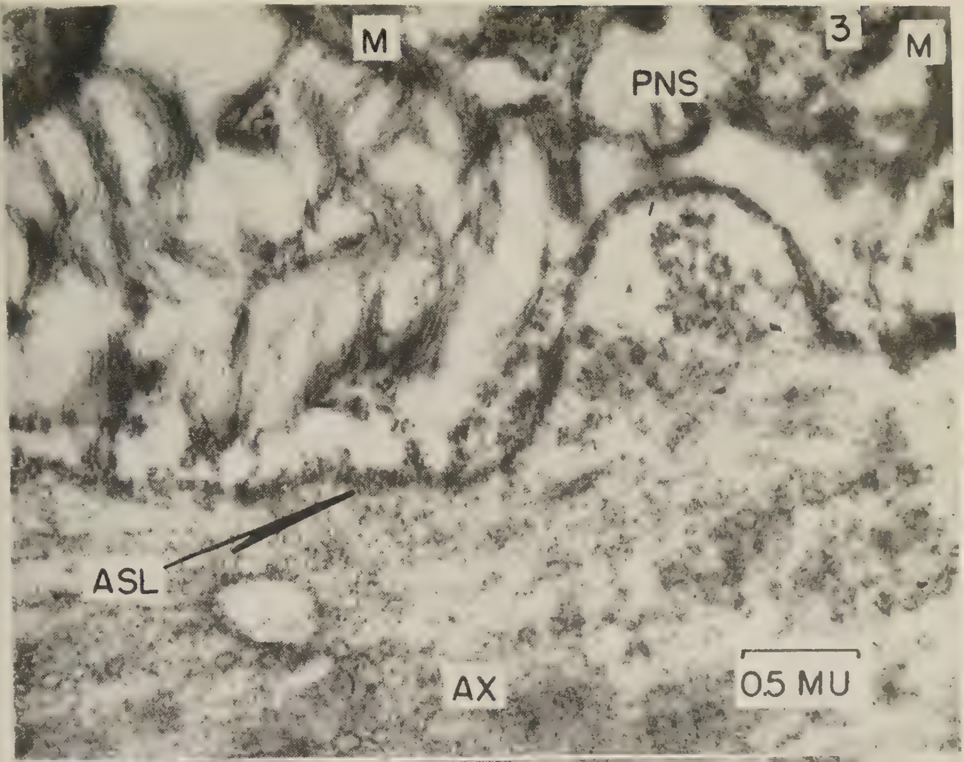
PLATE 3

EXPLANATION OF FIGURES

3 × 30,500. Same fiber as figure 2. Fibrillar and vesicular or annular structures in the axoplasm are evident, and the tendrils connecting myelin sheath and axoplasm are demonstrated. The axoplasmic surface layer is thick and appears irregularly discontinuous.

4 × 74,000. Myelin lamellae peeling off and turning in to approach axoplasm surface. Some of the terminal bulbs are faintly visible.

5 × 64,000. Vesicles 450–600 Å in diameter are demonstrated just inside the nodal axoplasmic surface. Larger bodies in the perinodal space are again seen.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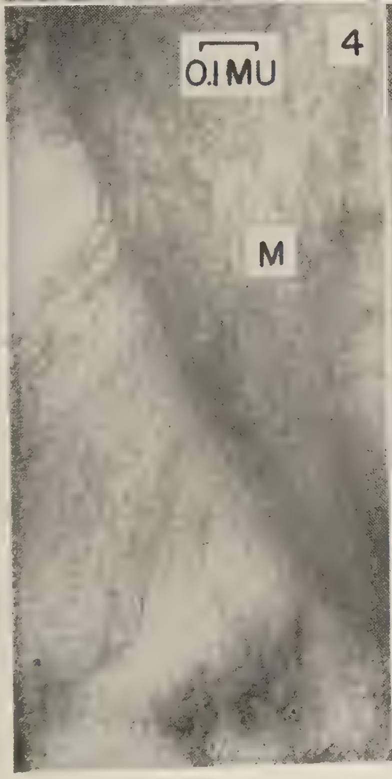
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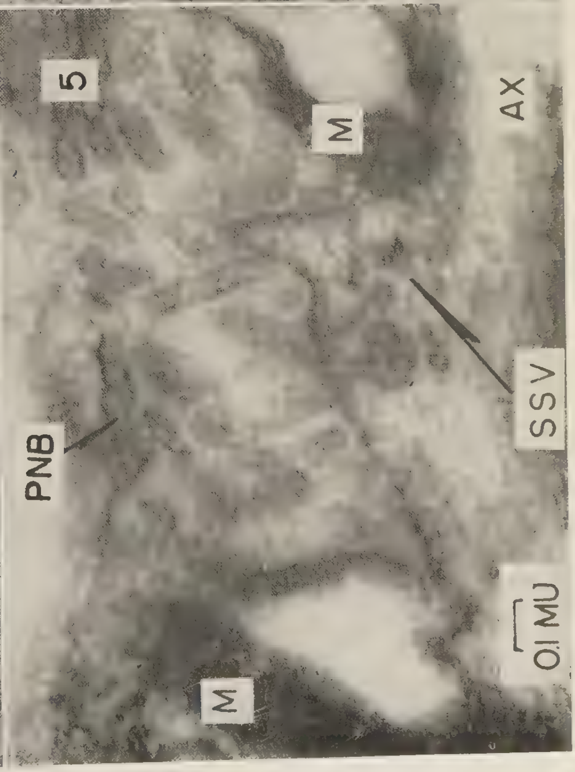
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SEDIMENTABILITY OF MICROSOMAL NUCLEOPROTEIN FROM RAT LIVER HOMOGENATES IN IONIC MEDIA[1]

I. PRECIPITATION OF PENTOSE NUCLEOPROTEIN BY $CaCl_2$.[2]

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Biology Department, New York University, New York City

FIVE FIGURES

Recently, Swedish (Hultin, '50a, '50b) and American (Gross, '54, '56) workers have shown that the addition of $CaCl_2$ in low concentrations to sea urchin egg homogenates initiates a series of reactions paralleling very closely those which, *in vivo*, accompany fertilization, sol-gel transformations, and the so-called "surface precipitation reaction" of injury (Heilbrunn, '52). Among these reactions is an aggregation process involving macromolecular materials and evidenced by greatly enhanced sedimentability of protein and pentosenucleic acid from the homogenates. In a previous publication, one of us (Gross, '56) has discussed evidence that the aggregating fraction is a nucleoprotein particle whose physical properties place it among the PNA-rich particulates of the microsome fraction. It has been suggested that this Ca-initiated reaction sequence, culminating in viscosity increase, acid formation, increased Q_{O_2}, and lysis of certain cytoplasmic inclusions, is either identical with or related to the normal sol-gel transformation mechanism *in vivo*.

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[1] Aided by a grant from the American Cancer Society.

[2] Some of the data reported here have been the subject of preliminary notes: Fed. Proc., *15:* 82 ('56), and J. Cell. and Comp. Physiol., *49, Supl. 1:* 243 ('57). Some of the results are taken from a thesis submitted by William Pearl to the Graduate Faculty of New York University, in partial fulfillment of the requirements for the degree of Master of Science.

Now this transformation is a property of every cell in which its study has been possible, and hence, our suggestion concerning mechanism would be greatly strenghthened if it could be shown that the Ca-initiated reactions of sea urchin egg breis are duplicable in materials from widely different animal sources. As a first step in such a program of comparative research, rat liver was chosen for the present series of experiments. The advantages of this material in terms of pre-existing biochemical information are evident.

A considerable literature exists already in the area of nucleoprotein solubility in electrolyte solutions, and from this it was clear that at least some Ca^{++} effect would be found. Indeed, Schneider ('46) has already shown that $CaCl_2$ precipitates pentose nucleoprotein from distilled water homogenates of rat liver.

In no case, however, has the effect been studied in homogenates of suitably perfused tissues, and in solutions of ionic strength and composition suitable for comparisons with the sea urchin egg data, and for discussions of what the authors consider possible physiological significance.

The experiments reported here constitute, in short, an attempt to extend the previous studies on homogenates of marine invertebrate eggs to properly prepared homogenates of a biochemically and physiologically well-characterized mammalian tissue; they were also designed to help in the evaluation of several environmental parameters influencing nucleoprotein solubility and in the identification of the sensitive fraction(s).

METHODS

Locally bred albino rats, weighing from 245 to 355 gm., were used. The animals were stunned by a blow on the head, immediately decapitated with a sharp meat cleaver, and allowed to bleed for a few seconds while suspended by the tail. The livers were perfused according to the method of Anderson ('55). This technique allows blood-free livers to be excised within 1–3 minutes after the beginning of perfusion. The perfusion fluid was an ice-cold mixture of equal parts of calcium-free Locke's solution and isotonic sodium citrate. Perfusion was begun usually not later than 50 seconds after decapitation, and the liver removed when blanching was complete, with perfusion maintained during excision. The livers were immediately weighed in a tared beaker of cold, isotonic KCl, blotted, and transferred to a chilled homogenizer containing the desired volume of isotonic KCl, the medium used for these experiments.

Homogenization was accomplished in a manually operated Potter-Elvehjem homogenizer with teflon pestle, modified in our shop for manual use. With this instrument homogenization was ordinarily completed with 20 strokes (up-and-down) of the pestle. The homogenate was stained through 4 layers of cheesecloth and finally centrifuged while ice-cold at 1000 rpm for 7 minutes in an "International" type SB centrifuge. The sediment contained the nuclei and a great many intact cells. Microscopic observation of the supernatant showed it to be essentially free of these contaminants.

In all experiments, the concentration of brei was one gm. (wet) of liver in either 4 or 5 ml of medium.

The design of most of the experiments was as follows: contamination-free homogenate was treated with $CaCl_2$ to give a final concentration of 0.025 M, except where this quantity was itself the variable. Controls were treated with KCl to yield solutions with final volumes and ionic strengths equal to those in the experimentals. All samples were then incubated at 37°C for 90 minutes, except in the kinetic experiments, where the incubation interval was variable. After incubation, the reactions were stopped by addition of ice-cold distilled water in sufficient volume. The diluted and chilled samples were then centrifuged at high speed in an angle centrifuge (Custom Scientific Instruments) on a specified schedule of acceleration and deceleration, with maximum R.C.F. of 16,500 g maintained for 5 minutes. The supernatants were then carefully separated from the sedimented material, and aliquots, suitably diluted, were analyzed for total protein, PNA, and in a special series, for Ca^{++}.

The supernatants were treated with trichloroacetic acid and allowed to stand, and the precipitates collected and processed according to the method of Schneider ('45), to separate protein and PNA for analysis. Pentose was determined by means of the orcinol reaction, and protein by means of a quantitative biuret reaction, the technique slightly modified from Fine ('35). This method, with crystalline ovalbumin as standard, served adequately for the comparative measurements required.

Acid formation was studied by withdrawal of samples from reaction mixtures at intervals, followed by measurement of pH in a Beckman model G pH meter. Similar and simultaneous runs were made with the KCl-treated controls.

Ca determinations were performed according to Horner ('55), a method which employs EDTA as a titrant for Ca^{++} in the presence of murexide (ammonium purpurate) as indicator.

When pH was to be kept constant in the reaction mixtures, tris-(hydroxymethyl)-aminomethane was used as buffer at pH 7.1.

In most experiments, absorption spectra in the ultraviolet region were obtained for the supernatants, suitably diluted. These spectra served both as a check on, and a complement to, the chemical analyses. The spectra were prepared with the aid of Beckman model DU or DK-2 spectrophotometers.

RESULTS

1. Ca^{++} concentration and nucleoprotein sedimentability. The homogenate contained one gram of liver in 4 ml of isotonic KCl. The final concentrations of $CaCl_2$ were varied. The result of the experimental procedures described in the *methods* section is shown in figure 1. PNA and protein in the untreated control were here taken as 100%, and the curves show protein and PNA *remaining unsedimented* in the experimentals after centrifugation. At concentrations higher than 50 Mm, no additional effect was observed, most of the PNA and about 35% of the soluble protein having become sedimentable. At all concentrations tested below 50 mM Ca^{++}, some increased sedimentability of PNA and protein was observed. The similarity in shape of the two curves shown in figure 1 may be interpreted tentatively as indication that a single particle or molecular type is being affected, one which contains both protein and PNA, and whose PNA accounts for most of the nucleic acid in the control systems (all the DNA is found in the sediments from the first purification step, *vide supra;* methods). It may be noted that significant effects

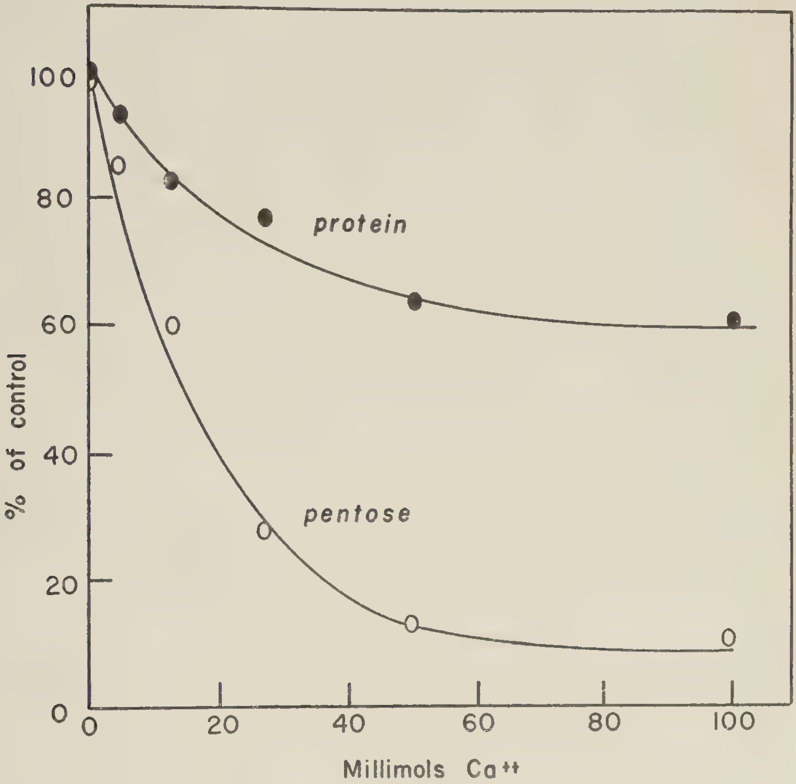

Fig. 1 Protein and pentose in supernatants from homogenates centrifuged at 16,000 g, after 90 minutes incubation at 37°C with varying concentrations of $CaCl_2$. Controls (100%) were treated with KCl to equal ionic strength. The curves are empirical.

were obtained in the physiological concentration range for Ca^{++}, and that in the range 0–20 mM, slight changes in the effective Ca^{++} concentration produce large effects on the state of aggregation of the homogenate nucleoproteins.

2. Kinetics of aggregation at normal ionic strengths. In this series, the homogenates contained one gm of liver in 5 ml of isotonic KCl. The Ca concentration was 0.025 M in experimentals, and the final ionic strength in both experimentals and controls was 0.228. This value is called "normal" because it corresponds to that for an isotonic uniunivalent salt solution containing, in addition, 0.025 M $CaCl_2$. The description "normal" is used to contrast the present results with those for systems at low ionic strength, *vide infra.* Maximum R.C.F. in the centrifugation step for this experiment was 16,000 g.

The results are shown in figure 2, with percentages of control initial values of PNA and protein plotted against the left-hand ordinate. There is found a sharp precipitation of protein and PNA in the first hour of incubation, the reaction continuing with decreasing velocity thereafter. These data show somewhat lower maximum relative precipitation effects than those seen in the series discussed above and shown in figure 1. The KCl-treated controls, from which divalent cations are absent (shown by titration of a control supernatant with EDTA), give no appreciable aggregation of nucleoprotein even after two hours. An incidental aspect of these findings concerns the usual experience of "aggregation" reactions in ionic media reported by many workers with tissue homogenates, and leading, among other causes, to the employment of nonelectrolyte solutions for homogenization. Such aggregations are probably most often initiated by the Ca^{++} remaining in unperfused tissues or by the divalent cations present in the usual ionic extracellular media.

3. Calcium binding by sediments. The Ca^{++} content of the supernatants from the experiment described in section 2 was determined by the method of Horner ('55), slightly modified. The results are shown in figure 2, with micrograms of Ca per aliquot plotted against time and with reference to the right-hand ordinates. All measurements were in triplicate, and the ranges of titer values are shown by the height of the vertical bars at the data points. The continuous dotted line is drawn through the mean values. In our hands the method gave useful, but rather imprecise results. The bar filled with slant lines shows the range of values for the calcium solution added to the experimentals. Although the spread of values is unfortunately large, it would be safe to conclude that experimental values falling within the bar should indicate that no binding of Ca^{++} had taken place, and means falling significantly below this region should indicate binding by sedimented material.

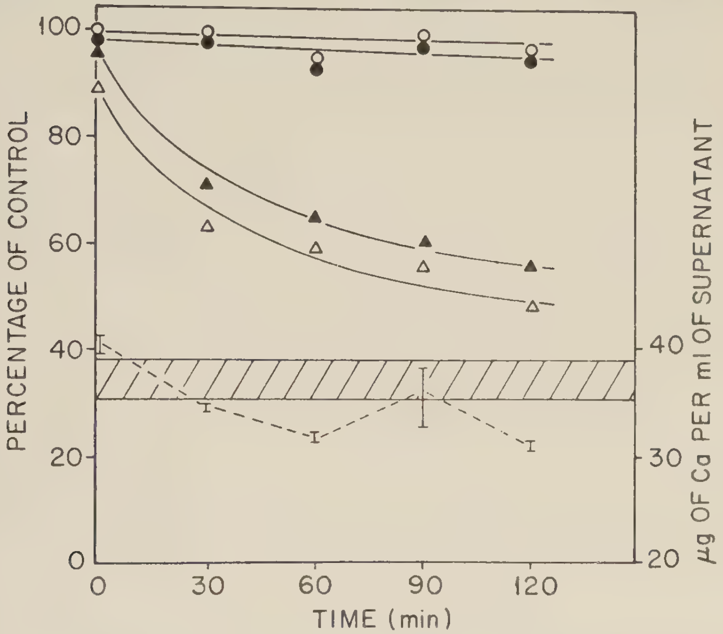

Fig. 2 Time course of precipitation of protein and PNA from rat liver brei after treatment with 0.025 M $CaCl_2$, and loss of Ca^{++} from 16,000 g supernatants. Left ordinates: ○, control (no Ca^{++}) protein, ●, control PNA; ▲, experimental protein, △, experimental PNA. Right ordinate: μg of Ca/ml of aliquot from supernatants. Shaded bar shows region of no binding by sediment: vertical lines show range of triplicate titrations; dotted line drawn through means. This figure has appeared in a preliminary discussion of this work. See footnote 2.

The results show a slight binding, on this basis, of Ca by the sedimented material, increasing with time. The degree of binding is, however, small, and may be of a nonspecific type; i.e., the ligand may be homogenate material other than the small nucleoprotein particles whose sedimentation behavior is under study. It is difficult to make estimates concerning how much Ca^{++} should be bound were the reaction mechanism a simple charge-neutralization on the sensitive macromolecular or particulate material; nevertheless, since 0.025 M Ca^{++} is well below the concentration for maximal effect in the intervals studied, it might be concluded that all sites might not be occupied in such a simple double layer affect, and that hence appreciable binding should have been observed. In other terms, these results (i.e., about 14% maximal binding of available Ca^{++}) do not necessarily support a direct discharge mechanism for the aggregation reaction, nor can they be invoked in these experiments in favor of an indirect (say, enzyme activation) mechanism. The problem is soluble only if the sensitive fraction is exposed, free of other substances normally found in the homogenate, to Ca of known concentration, and most favorably, containing a known specific activity in Ca^{45}; after which binding and precipitation could be studied with sufficient sensitivity. Such an experiment is being developed in this laboratory at the present time.

4. *Absorption spectra.* Figure 3 shows the absorption spectra in the ultraviolet region for the supernatants analyzed chemically in the experiments described in section 1. They are replotted aganst a linear wavelength abcissa from continuous curves drawn by the Beckman model DK-2 spectrophotometer.[3] Aliquots of the supernatants from experiments in section 1 were diluted for the optical measurements. The pH was 6.8 in all samples. "X" is the spectrum of the control supernatant, and the other curves are for homogenates treated with various concentrations of $CaCl_2$. The Ca^{++} con-

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[3] We wish to thank Dr. Thomas Freund, of the Chemistry Department, University College, New York University, for his assistance in obtaining the use of the DK-2 spectrophotometer.

centrations obtaining in the various samples were as follows:

"A"	0.1 M
"B"	0.05 M
"C"	0.025 M
"D"	0.0125 M
"E"	0.005 M

The important changes in these spectra, with increasing sedimentation of the sensitive nucleoprotein fraction, are:

1. decrease in absorbancy at 280 mμ

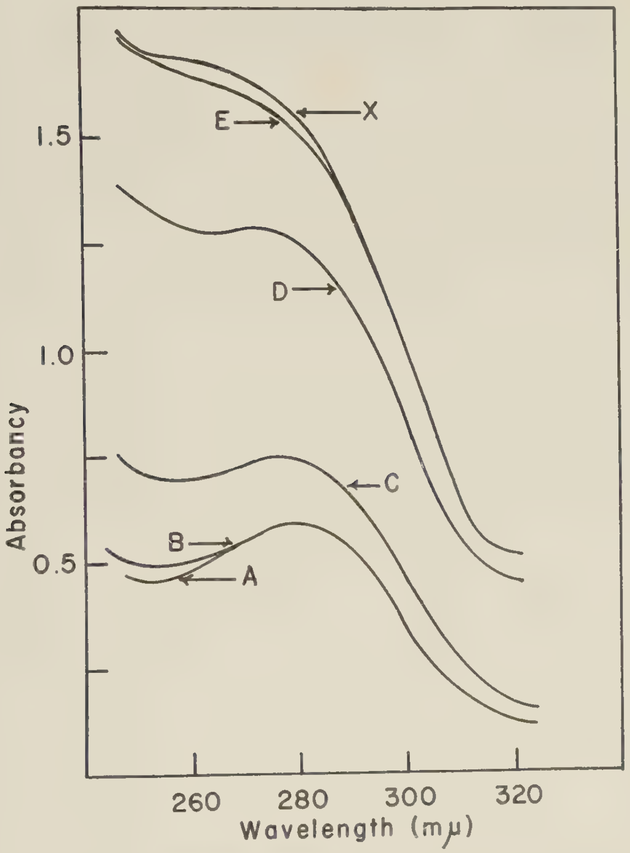

Fig. 3 Ultraviolet absorption spectra of diluted supernatants from experiment 1 (see text and fig. 1). Spectra replotted, against linear wavelength abscissa, from continuous spectra recorded on Beckman model DK-2 spectrophotometer. "X"; control, no Ca^{++}. "A"-"E", experimentals, treated with Ca^{++}. See text for concentrations.

2. a larger decrease at 260 mμ
3. appearance of a minimum at 240–250 mμ.

These changes suggest that:

1. the material being lost in sedimentation contains both protein and nucleic acid
2. this fraction accounts for most of the PNA of the homogenate (note shift in the maxima from 260 to 280 mμ with increasing sedimentation)
3. the precipitating material contributes, in the controls, very greatly to the light scattering of the samples; this effect is, in all likelihood, a result of very large particle size, even with respect to ordinary molecular dimensions for proteins.

Suggestion 3, taken together with the fact that the sensitive material is nevertheless sufficiently small to escape sedimentation at 16,000 g in the controls (a speed which sediments all mitochondria, cf. Gross, Pearl, and Moss, '58), would appear to localize the sensitive material to the microsome fraction or to a PNA-rich, high molecular weight nucleoprotein particle of the "soluble phase."

5. Acid formation. Addition of Ca^{++} to sea urchin egg homogenates evokes production of acid; a similar event follows fertilization, stimulation, or injury of the living cell. To test the liver homogenate for the presence of this effect, two samples of a 1:5 homogenate were obtained; one was treated in the usual way with Ca^{++}, the other with KCl; pH values of aliquots of both samples were determined at intervals with a Beckman model G pH meter. The result is shown in figure 4. The pH of the Ca-treated sample fell from 6.84 to 6.62 within 25 minutes, with most of the acid production within the first 5–10 minutes. Such a pH drop indicates a very considerable production of protons, since the homogenate is strongly self-buffering at the initial, or control pH. The maximum expected change in pH due to the addition of slightly acid $CaCl_2$ solution is 0.07 pH, with no buffering. If proton release can be interpreted here as resulting from binding of Ca^{++} to undissociated acidic side-chains of the sensitive macromolecule or particle, it might be supposed that this phase of the reaction is complete well before the (thermal ?) aggregation becomes detectable.

6. *Isoelectric precipitation?* The change in pH accompanying Ca treatment of homogenates might be responsible for the observed aggregation of nucleoprotein, for such an increase in acidity would lower the net charge on polyanions, permitting closer approach and possibly precipitation. To test this possibility, two $CaCl_2$-treated samples and two KCl-treated samples were prepared. One sample of each pair was buffered with "tris" at pH 7.1. The centrifugation and assay procedures were then followed as usual. Table 1 gives the results of this experiment. These results are in accord with those obtained previously for sea urchin egg breis. Within the limits of error of the analyses, there is no significant difference in the extent of aggregation between buffered and unbuffered systems. It is necessary to conclude from this observation that the sensitive fraction does not aggregate

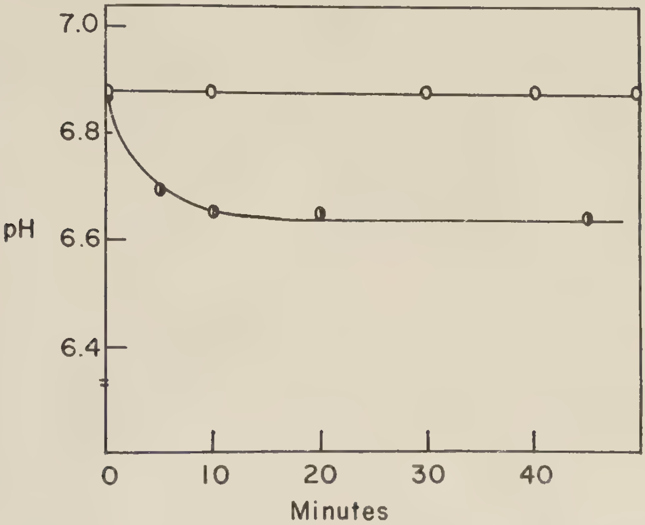

Fig. 4 Acid formation in liver homogenate (1 gm liver in 5 ml isotonic KCl) after addition of 0.025 M $CaCl_2$. pH read with glass electrode. Open circles: control (KCl); half-filled circles: experimental ($CaCl_2$).

simply because the addition of Ca^{++} lowers the pH, but rather that aggregation results from a specific interaction, direct or indirect, between the sensitive particle and the divalent cation; in fact, it is not unreasonable to expect that the pH drop is a direct result of the interaction. In view of the important role known to be played by Ca^{++} in both fertilization and injury processes, it may be considered, as a basis for further work, that the acid formed concomitant to these processes arises from the same interaction.

TABLE 1

SAMPLE TREATMENT	μG PENTOSE IN ALIQUOT OF SUPERNATANT
KCl, buffered	42.0
KCl, unbuffered	44.3
$CaCl_2$, buffered	19.1
$CaCl_2$, unbuffered	17.1

7. *Ionic strength.* A homogenate preparation free of nuclei and whole cells was obtained and dialyzed against distilled water at pH 6.0 for 18 hours at 0°C. The water was changed frequently. The dialyzed material was subsequently centrifuged at 1000 g to remove precipitate and debris, and the floating fat layer was removed by suction. Eight tubes were prepared, each containing 2 ml of dialyzed homogenate and 2 ml of a KCl solution whose concentration was systematically varied. To each tube, one ml of a 0.025 M $CaCl_2$ solution was added, providing a final concentration of 0.005 M Ca^{++} in each sample. The final concentrations of KCl were 0, 0.015, 0.061, 0.248, 0.333, 0.500, 0.748, and 1.0 M. Immediately upon addition of the $CaCl_2$, a finely divided white precipitate appeared in the tubes with solutions of low ionic strength (0 to 0.061 M KCl). At ionic strengths near ''normal'' and higher, no precipitate formed. Thus the rapid precipitation observed here could not have occurred in the systems discussed in previous sections: these were all in salt equal to or greater than 0.15 M KCl. Figure 5 shows the

results of analyses performed on the supernatants from the samples described above; each sample was centrifuged (after 15 minutes of incubation at 37°C) in the high-speed centrifuge at a R.C.F. of 2,500 g. The precipitates were discarded, and PNA and protein measured in aliquots of the supernatants. In the figure, the left-hand ordinates and open circles represent PNA, and the right-hand ordinates and filled circles represent total protein. Clearly, the rapid precipitation reaction cannot occur at physiological ionic strengths.

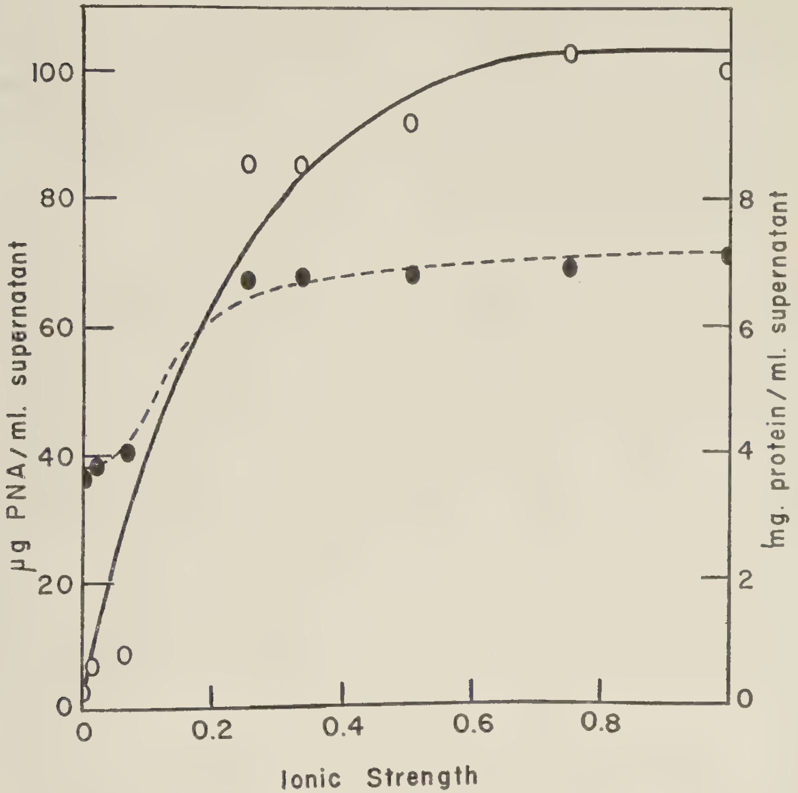

Fig. 5 Rapid precipitation of nucleoprotein from dialyzed homogenate with ionic strength subsequently adjusted. Each sample contained 0.005 M Ca^{++}, and KCl to vary the total ionic strength from near zero to 1.0. After 15 minutes, all samples were centrifuged at 2,500 g, and the supernatants analyzed for total protein and PNA. Left ordinates: μg PNA/ml supernatant; right ordinates: mg protein/ml supernatant. The curves are empirical.

One recognizes the possibility that the "instantaneous" reaction involves a fraction different from the one which aggregates slowly at normal ionic strength, or that two species are involved, the "instantaneous" precipitate bringing down some of the "slow" material. The simplest explanation is, of course, that the material which aggregates slowly at high ionic strength does so instantaneously at low; but such is not the case in sea urchin eggs, where a soluble phase macromolecule, normally stable in $CaCl_2$ at high ionic strength, precipitates at low (Gross and Nass, unpublished experiments). There is reason to believe, from the relative quantities of material precipitated at low and at high ionic strength after incubation to equilibrium, that in liver brei, a fraction precipitates in solutions of very low salt content with Ca^{++} which is indefinitely stable at high salt concentrations with the same quantity of Ca^{++}.

DISCUSSION

Addition of Ca^{++} to Ca-free sea urchin egg homogenates elicits the following results: increased oxygen uptake, viscosity increase, acid formation (Hultin, 50a, '50b), lysis of cytoplasmic inclusions (Hultin, 50a, Gross, '51, '54), and aggregation of nucleoprotein (Gross, '54, '56). The origin of the nucleoprotein appears to be in submicroscopic particulates corresponding, in this cell, to the PNA-rich "dense" particles of the microsome fraction. One of us (Gross, '56, '57) has considered this system, as a working hypothesis, an *in vitro* model of the surface precipitation reaction (Heilbrunn, '52) and of the cytoplasmic sol-gel transformation.

The experiments reported here show that in most respects, the reactions observable in sea urchin egg breis can be reproduced in rat liver homogenates, a fact which argues for the widespread character, and hence the utility as a model, of this set of processes. More specifically, the following observations are now known to coincide for both liver and sea urchin eggs: (1) Ca^{++} added to breis prepared from Ca^{++}-free cells initiates nucleoprotein aggregation; (2) the process is slow at physiological ionic strengths; (3) it is accompanied by increased proton activity; (4) it is a cause, rather than a result of, this increase in activity, and finally (5) at low ionic strengths, a new, rapid nucleoprotein precipitation reaction is elicited (for sea urchin eggs, Gross and Nass, unpublished).

These results may be compared profitably with those of Schneider ('46), who reported a careful study of precipitation of pentose nucleoprotein from distilled water homogenates of rat liver. Schneider observed instantaneous precipitation rather than the slow aggregation described by Gross ('56) in sea urchin eggs and now, at high ionic strengths in liver. The former author was able to precipitate essentially all of the PNA at near-physiological concentration levels of $CaCl_2$, i.e., 0.033% as compared with the normal serum level of 0.027%. Reference to the ionic strength effect herein described resolves the apparent discrepancy. Schneider's homogenates were made in distilled water and without perfusion of the tissue. Our own observations on dialyzed homogenates confirm Schneider's in all respects.

One of his observations is, in this regard, particularly significant: when NaCl was added to the homogenates along with $CaCl_2$, the rapid precipitation reaction was inhibited, so that at physiological level (0.9% NaCl), the yield of both protein and PNA in the precipitate was sharply lower. We consider that had Schneider's material been perfused, and thus freed of fibrinogen and the normal tissue Ca^{++}, the yield in 0.9% NaCl would have fallen close to zero for the "instantaneous" precipitation.

The rapid precipitation at low ionic strength can be considered from the point of view of kinetics if the yield of precipitate over a constant short interval is treated as a rate. If this is done with our data, one finds the rate of precipitation declining exponentially with increasing total ionic strength (KCl), while the concentration of initiating ion (Ca^{++}) remains constant. Such a relation recalls the primary kinetic salt effect, first discussed by Brønsted, and formalized in the relation:

$$\log_{10} k = B + 1.018 \; z_a z_b \sqrt{s}$$

where k is the rate constant, B is a constant containing (as is now understood) $(kT/h)\cdot K^{\ddagger}$, the z's are the charge numbers of the reactant ions in a bimolecular process, and s is the *total* ionic strength. This relation predicts that at 25°C in water, in a reaction between two oppositely charged ions, the rate will decrease with increasing total ionic strength, since the charge product is negative; in fact a plot of log k against $\sqrt{s}$ will be linear with slope equal to the charge product.

The data presented here are inadequate to justify a complete analysis in terms of the primary kinetic salt effect, but it may be concluded, tentatively, from the approximate fit of the data to the Brønsted relation, that the precipitation observed in distilled water homogenates does involve a direct interaction between an anionic macromolecule and the cationic Ca^{++}. Whether this analysis applies to the reaction observed at high ionic strength must, for the moment, remain undecided. The aggregating material, in any event, at high ionic strength, is microsomal in both sea urchin (Gross, '56, Gross and Nass, unpublished) and in liver (see also Gross, Pearl, and Moss, '58): in sea urchin, a soluble phase nucleoprotein which is stable at physiological ionic strength accounts for the instantaneous precipitation with Ca^{++} in water, and the quantitative relations between equilibrium precipitates at both high and low s suggest that the same is true for liver homogenates.

It has been demonstrated, therefore, that on the score of widespread distribution, the constellation of Ca-induced reactions in homogenates remains a useful working model of the *in vivo* biochemistry and colloid chemistry of injury (surface precipitation reaction) and of sol-gel transformations; at least the fundamental character of the process is evidenced by its presence in such widely different cells as sea urchin eggs and perfused rat liver. The literature of cellular physiology provides ample justification for the basic experimental motivation, i.e., release of divalent cations into a macromolecular-particulate system bathed by a medium whose cations are normally inorganic and univalent. (cf. Anderson, '56).

SUMMARY

Homogenates prepared from rat liver perfused with a Locke's solution-isotonic citrate mixture have been treated with Ca^{++} at varous concentrations and at a series of values of total ionic strength. In the region of "normal" ionic strength, i.e., 0.15–0.25 M uniunivalent salt, there is observed a slow, continuous aggregation of nucleoprotein, manifested by sedimentability at 16,000 g of particles normally not sedimented at this R.C.F. When the ionic strength is low, i.e., below 0.06, addition to the homogenates of very low concentrations of Ca^{++} (0.005 M) elicits an instantaneous precipitation of white, finely divided material. This precipitate contains almost all of the PNA of the homogenate. Both precipitation reactions involve cell fractions comprising particles of microsomal or smaller size. The slow reaction at normal ionic strength, and hence the one of physiologic importance, appears to involve the PNA-rich small particles of the microsome fraction almost exclusively. The aggregation reactions are accompanied by a large increase in protonic activity, and it is suggested that this reaction is, at least at low ionic strength, due to binding of divalent cations at undissociated acidic sites on the macromolecules or particles which sediment. The aggregation is a cause, rather than a result, of the pH drop. It proceeds in strongly buffered systems at pH 7.1.

These observations duplicate results obtained for homogenates of sea urchin eggs. The aggregation process has been interpreted as an *in vitro* model of the cytoplasmic sol-gel transformation.

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THE EFFECT OF TEMPERATURE ON POTASSIUM TRANSFER IN IRRADIATED YEAST[1]

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FOUR FIGURES

Studies of potassium leakage from yeast following x-irradiation have shown a loss in the ability of the cell to retain this cation. The magnitude of this increased loss has been shown to be dose-dependent (Bair, Stannard and Bruce, '56) and also influenced by the carbon source utilized for metabolic energy during the measurement of the potassium leakage (Bruce and Stannard, '58). The ability of the cell to retain potassium appears to be in some way linked to metabolism and some alteration in this linkage occurs as a result of exposure to ionizing radiation. The defect may relate primarily to the rate of efflux from the cell, to potassium uptake or involve both. It is obviously dependent in part on the nature of the substrate. The present study attempts to relate the response of both components of this cationic exchange to alterations in the metabolic activity of the cell brought about by temperature changes. While direct correlation with specific metabolic processes is not possible by this method, general relationships can be found. These include a clear indication that the effects of irradiation on the two processes, uptake and leakage, are in opposite directions and to a degree separable.

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[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York. Some of it appears in more detail in a thesis by A. K. Bruce submitted in partial fulfillment of the requirements for the Ph.D. degree, Departmnt of Radiation Biology, University of Rochester, 1956.

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MATERIALS AND METHODS

The yeast used in this investigation was obtained from Standard Brands, Inc. Portions from the center of the block were washed three times by suspension in an equal volume of distilled water followed by centrifugation. The yeast was then starved for three hours by aeration of a suspension of approximately 300 mg/ml (wet weight), then centrifuged and resuspended before use. The yeast concentration was determined by turbidimetric methods (Bair and Stannard, '55).

Radiation procedure. A yeast suspension of 10 mg/ml in distilled water was divided into two portions of 250 ml each, one being irradiated and the other serving as control. Each suspension was placed in a crystallizing dish of 125 mm diameter resulting in a depth of 20 mm and was bubbled with oxygen during the irradiation period. A dose of 60,000 r was delivered to the yeast by a Picker Industrial x-ray unit operated at 250 Kvp and 15 ma. Only inherent filtration was used and the center of the suspension was at a distance of 6 5/8 inches from the target. The resulting dose rate was about 1300 r/minute as determined with a 1000 r Victoreen Thimble chamber in place of the sample dish.[3] Radiation was done at room temperature (25°C).

Potassium exchange procedure. Potassium transfer in the yeast was studied by the method of column elution (Rothstein, '55, '56). After irradiation 500 mg of yeast were transferred to a Buchner-type funnel of 30 mm diameter having a fine porosity fritted disc and sucked into a cake. A sheet of filter paper and some glass wool were placed above the yeast to prevent agitation. Test solutions containing either 0, 5×10^{-4} or 1×10^{-3} M potassium at a pH of 3.5 either with or without added glucose or fructose, 0.05 M, were passed through the yeast at 6 pounds pressure. The solutions as passed through the yeast columns were held at various temperatures by placing the vessels containing them in either a refrigerated box, ice bath or water bath covering a temperature range of 1 to 36°C. The temperature of the solution in contact with the yeast was measured with a thermometer inserted into the top of the column containing the yeast. Samples of the effluent were collected on a time basis which varied from 2.5 to 30 minutes depending upon experimental conditions. The potassium content of these samples was determined by flame photometry.

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[3] The irradiation and dose rate measurements were carried out by Mrs. Florence Van Slyke and Miss Patricia Ewart of the Radiation Tolerance Section.

RESULTS

The rate of leakage of potassium into a potassium-free medium was found to increase with increasing temperature with all substrates studied. This was found for both irradiated and control yeast. Typical experiments are shown in figure 1. Rates of leakage, as determined from the slope of the most linear portion of the leakage curves, varied from 1 mM/kg/hr in unirradiated yeast undergoing endogenous metabolism at 1°C, to 60 mM/kg/hr in irradiated yeast measured with glucose the substrate at 36°C. It is apparent from the figure that the difference between irradiated and control yeast is relatively greater at low temperatures than at high temperatures both with and without added external substrate.

The rate of potassium uptake is illustrated in figure 2, which again shows typical experiments. Uptake is negligible in the absence of external substrate, and qualitatively similar with either glucose or fructose. The rate is dependent, in part, on the external potassium concentration, being greater at the higher concentration studied.

As seen in figure 2, uptake, like leakage, is considerably more rapid at higher temperatures. Radiation appears to *decrease* the rate of uptake in contrast to its effect on leakage. As in the case of leakage, the effect of radiation is relatively greater when measured at lower temperatures.

The uptake process decreases rapidly with time as the cell accumulates potassium. Thus the rates from the most linear portion of the initial uptake phase are employed for further

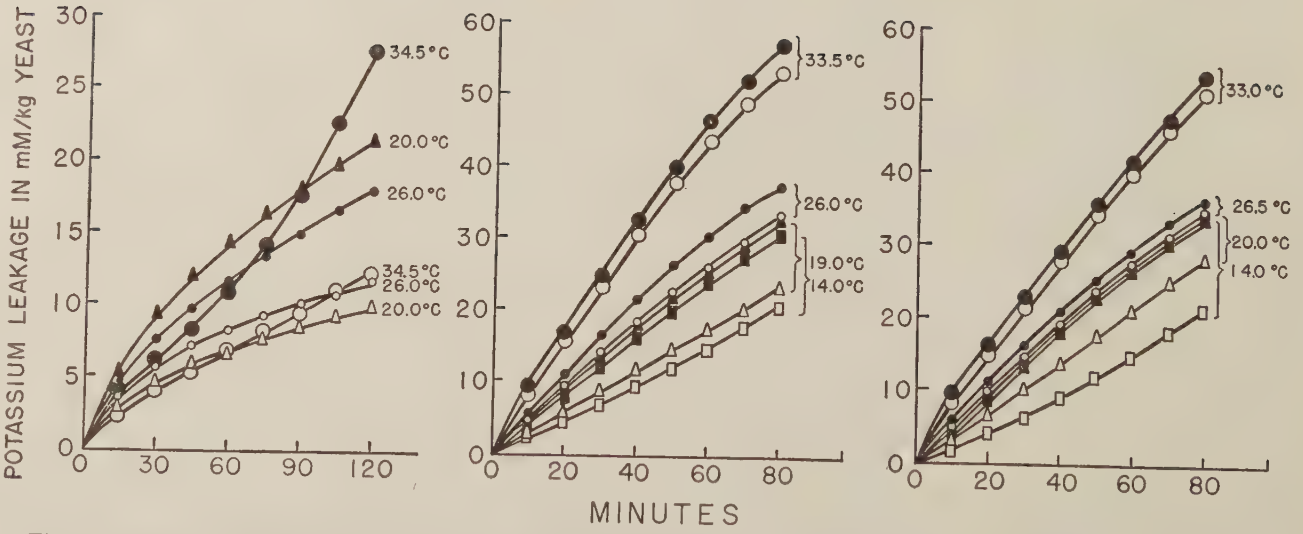

Fig. 1 Potassium leakage as influenced by temperature and substrate. Left, no added glucose; center, glucose added; right, fructose added. The solid points are from samples irradiated with 60 kr and the open points from unirradiated controls. The most linear initial portion of these curves was used for calculating an average rate of leakage.

analysis. Also, since the column method employed gives only the net change in rate of potassium accumulation with time, some correction must be made for the simultaneous leakage of potassium in order to arrive at a value for the true rate of influx of the cation. These calculations of influx have been made by adding the value obtained for leakage at the same temperature with the same substrate to the net accumulation

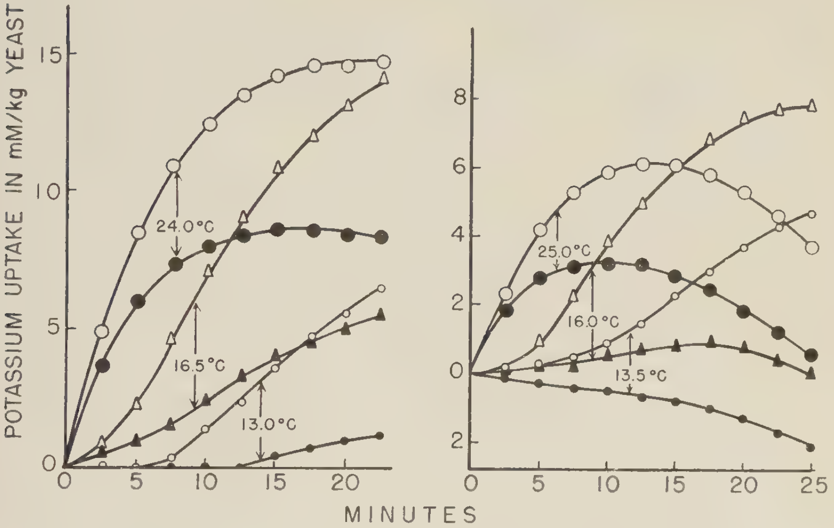

Fig. 2 Potassium uptake by respiring yeast with glucose the substrate at various temperatures. The external potassium concentrations are: left, 1×10^{-3} M and right, 5×10^{-4} M. The solid points are from samples irradiated with 60 kr and the open points from unirradiated controls. The most linear portion of the uptake phase was used for calculating the "mean" rates used in the text and subsequent figures.

of potassium in uptake experiments. The numbers used in each case can be seen on the ordinates of figures 3 and 4. Their summation results in calculated rates of inward flux of potassium between 15 mM/kg/hr and 162 mM/kg/hr depending on the temperature and the external concentration of cation.

The mean rates of both leakage and of uptake are plotted as a function of temperature in figures 3 and 4. The Arrhenius notation is used for reasons to be taken up in the discussion.

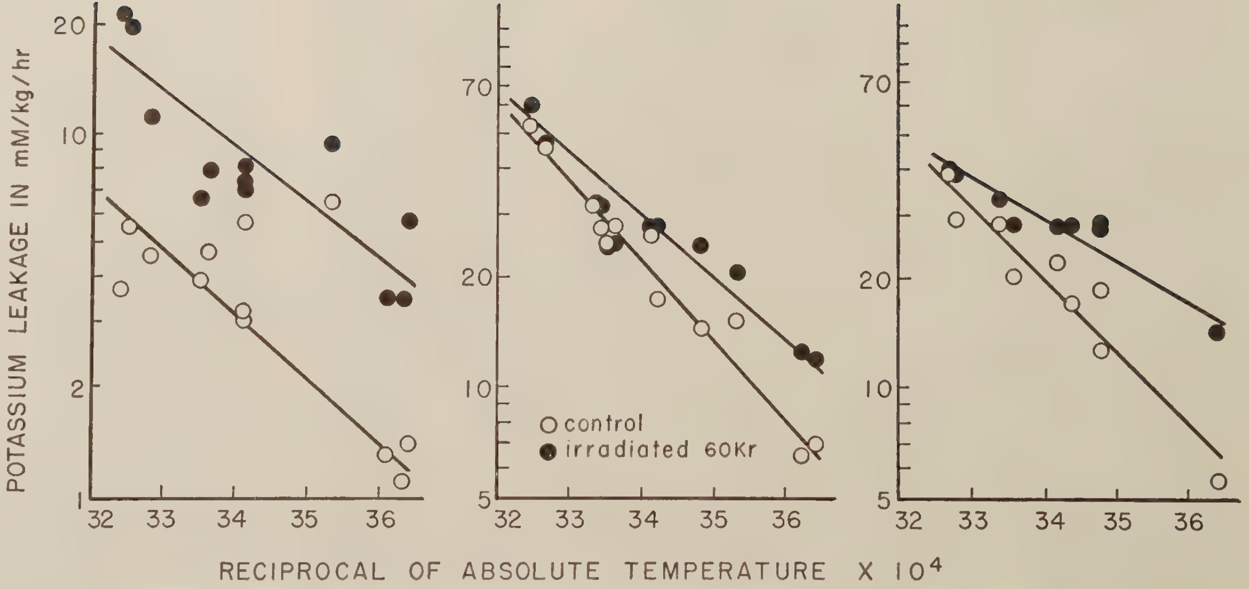

Fig. 3 Arrhenius plots for the effect of temperature on potassium leakage. Left, no added substrate; center, glucose added; right, fructose added.

The trend toward more rapid exchange in both directions as temperature is raised is clear from these plots. The radiation effect is also clear in that the slopes of the lines as well as their position is altered.

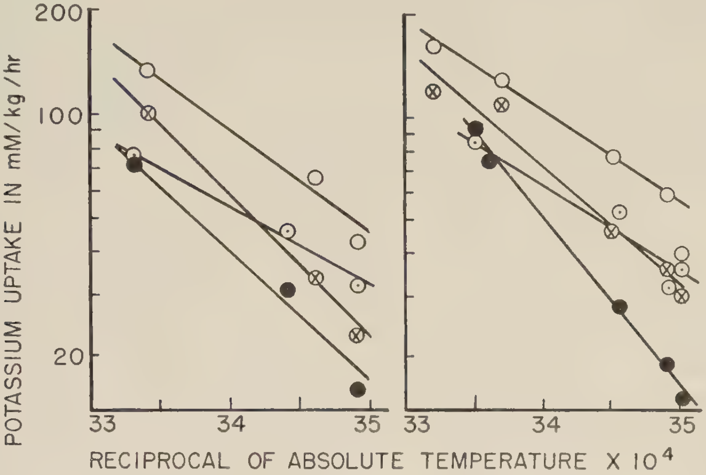

Fig. 4 Arrhenius plots for the effect of temperature on potassium uptake. Substrates are: left, glucose; right, fructose. Control: ◉, 5×10^{-4} M K^+ ○, 1×10^{-3} M K^+ Irradiated 60 kr: ●, 5×10^{-4} M K^+ ⊗, 1×10^{-3} M K^+

DISCUSSION

The effect of temperature on both uptake and leakage provides information pertinent to the possible mechanisms involved here as well as to the superimposed radiation effect. In figures 3 and 4, the changes in rates of leakage and uptake as a function of temperature have been plotted in accordance with the Arrhenius equation. When expressed in this manner the data can be fitted reasonably well by straight lines. The values of the "temperature characteristic," μ,[4] calculated from the slopes of these lines are summarized in table 1. The μ values calculated for both uptake and leakage of the control yeast are in the order of 10,000 cal/mol. Irradiated yeast gives a higher μ value for uptake with both glucose and fructose as substrates, averaging about 18,000 cal/mol, and a lower μ value for leakage, about 6,000 cal/mol.

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[4] This notation is used rather than activation energy to preclude any implication that the slope of the lines represents a true activation energy for a simple chemical system. (cf. Sizer, '43.)

Only leakage could be measured in the experiments without added substrate since uptake is negligible. The value of the Arrhenius constant appears slightly lower in the unirradiated yeast, but the fit of the line for endogenous leakage (fig. 3) is not as good as in experiments with substrate present because of the greater variability of measurements of these lower rates. The radiation effect is in the same direction as in the presence of external substrate.

TABLE 1

Average values for the Arrhenius constants (μ) [1] for potassium exchange in yeast

SUBSTRATE	EXTERNAL K+ CONC (M)	ARRHENIUS CONSTANTS IN CAL/MOL			
		Uptake		Leakage	
		Cont	Irrad	Cont	Irrad
Endogenous	0			8,000	6,300
Glucose	0			10,000	7,800
	5×10^{-4}	11,000	22,600		
	1×10^{-3}	12,100	15,800		
Fructose	0			9,100	5,300
	5×10^{-4}	10,300	16,700		
	1×10^{-3}	13,500	18,600		

[1] Sometimes referred to as a "temperature characteristic" for biological systems to prevent any implication that it represents a true activation energy.

The value of the "temperature characteristic," μ,[4] is of some help in differentiating between enzymatic and purely physical processes. Theoretically, μ represents the minimum energy, in calories per mole, that a system must attain in order for a reaction to proceed. It is often assumed that chemical or biochemical reactions have high "temperature characteristics," in the order of 12,000 cal/mol or higher, while physical processes have lower values of about 3,000 to 5,000 cal/mol (Sizer, '43). The uptake process, being against a concentration gradient, is of necessity an active process since energy is required for such a movement of ions. Leakage is with the concentration gradient. It may then be either passive or active. The experimentally determined values of μ of about 10,000 cal/mol for both uptake and leakage of potassium in the unirradiated yeast with external substrate present are compatible with requirements for the involvement of enzymatic reactions in both. While values of the "temperature characteristic" do not alone indicate whether a process is "active" or not, since uptake is known to be active and the values for uptake and leakage are nearly identical, it may be considered as presumptive evidence for a linkage of both processes to metabolism. It does not, however, eliminate the possibility of "activated diffusion" (Davson and Danielli, '43) in the leakage process.

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[4] See footnote 4, page 171.

The effect of irradiation upon the μ values is of considerable interest. The higher value for potassium uptake in irradiated yeast would indicate that, on the average, more energy is required for uptake of potassium after irradiation. The lower value for leakage obtained in irradiated yeast is almost at the level sometimes associated with a diffusion process. This would indicate that, although both uptake and leakage are normally linked to metabolism, irradiation places an increased demand upon metabolic energy for uptake, while leakage may revert to nearly a passive diffusion. In short, these studies indicate that at least one effect of irradiation upon the yeast cell results in a decoupling of metabolism from the processes involved in potassium exchange. Billen et al. ('53) have demonstrated that a loss of ATP occurs in bacteria following irradiation. This may be involved in changes in cell surface properties as studied here.

The changes in μ noted after irradiation point up the importance of considering the conditions of measurement in interpreting radiation effects. Since the effect of radiation is to *increase* leakage and *decrease* uptake while the μ values change in opposite directions, the net result is an apparently greater relative effect of radiation at the lower temperatures. Conversely, a temperature might be found at which radiation would apparently have no effect at all, though probably other factors would interfere if the temperature were raised to this point. It is interesting to note that Stapleton, et al. ('53) have shown that survival of *E. coli* after x-irradiation is dependent upon the temperature of incubation.

Further experiments to determine the effects of radiation on exchange of other ions, the effect of other forms of radiation, and studies of the mechanism of the changes in potassium retentivity are in progress or planned.

SUMMARY

1. The leakage and uptake of potassium in normal and x-irradiated yeast have been studied at temperatures from 1 to 36°C. Irradiation was carried out at 25° in all cases.

2. Both processes are increased in rate by increasing temperature and follow the pattern expected of "active" metabolic processes.

3. Radiation increases the rate of leakage but decreases the rate of uptake of potassium ion.

4. The relationship of the radiation effect to temperature indicates an increased temperature coefficient (as measured by the "temperature characteristic," μ, in the Arrhenius equation) for uptake, a decreased temperature coefficient for leakage of potassium. This results in an apparently greater relative effect of radiation on both processes at the lower temperatures.

5. It is possible that radiation accomplishes, in part, an uncoupling of the metabolic processes involved in active transport in the case of leakage, but makes the uptake process more dependent on energy expending reactions.

6. The need for consideration of the conditions of measurement in assaying radiation effects is pointed up by these experiments.

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REGENERATION OF LIMB ABNORMALITIES AFTER ULTRAVIOLET IRRADIATION[1]

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FIVE FIGURES

It was found in earlier studies that if, after irradiation of the forearms of larvae of the salamander *Amblystoma* with ultraviolet light, the limbs were amputated in the irradiated region, the regenerating limbs showed a high proportion of abnormalities (Blum et al., '57). It was also observed that by localized irradiation of the elbow region a high proportion of supernumerary appendages could be induced, having a variety of forms ranging from a single digit to a nearly complete hand (Butler and Blum, '55). With this ready means of obtaining abnormal appendages, it has seemed desirable to study regeneration after amputation of the abnormal parts. The primary question is: will the regenerate duplicate the pattern of the abnormality of the part amputated? The answer, in general, seems negative. Our results indicate, also, that amputation of the irradiated forearm may promote recovery toward the normal.

METHODS AND MATERIAL

There has been no essential change from the methods used in the previous studies referred to above. Since it was desired

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[1] This investigation was supported in part by a research grant, C-1499, from the National Cancer Institute, of the National Institute of Health, Public Health Service.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare.

to obtain as high proportions of abnormalities as possible, the animals were always kept in the dark after irradiation, in order to avoid photorecovery, which would have reduced the number of abnormalities or supernumeraries. The animals used were *A. punctatum* and *A. opacum,* obtained from the same sources as in the previous studies, and selected for uniformity in the same way.

RESULTS

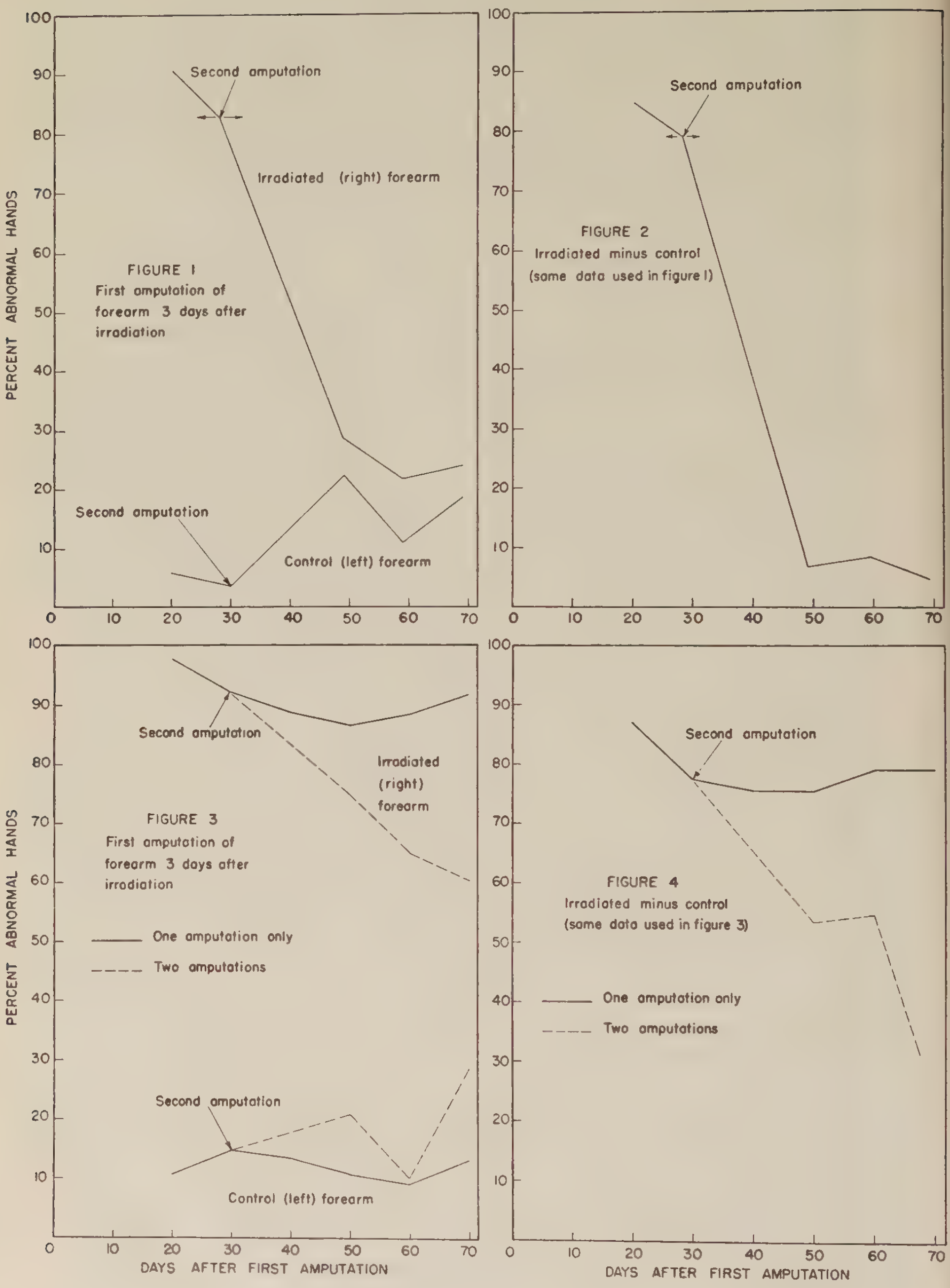
A first experiment (Series LA) was designed to examine regeneration of the irradiated forearm after amputation; 201 larvae of *A. punctatum* averaging 28 mm total body length (S.D. = 3.66) were used. The right forearm was exposed to a single dose of ultraviolet light, 6.2×10^7 ergs cm^{-2} of wavelengths 0.313 μ and shorter. This is the same dose used in previous studies of this kind (Blum et al., '57; Butler et al., '57), and is sufficient to produce a high proportion of abnormalities in the regenerates after amputation. Three days after the irradiation both forearms were amputated through the distal region of the radius and ulna, the unirradiated (left) forearm served for comparison with the irradiated (right) forearm, as in previous studies. It has been found in experiments with photorecovery (Blum et al., '57) that the proportion of forearms which develop abnormalities is fixed within the first few days after irradiation, and that up to 30 days there is little return toward the normal state. It was, therefore, elected to amputate a second time 30 days after the first amputation, when the character of the abnormality was clearly established in the regenerate. The level of the second amputation was dictated to a certain extent by the character of the abnormality which had developed after the first amputation; it was, however, always through the portion of the arm originally irradiated.

Figure 1 summarizes the proportion of abnormalities observed, at intervals following the first and second amputations. A slight tendency for the abnormal regenerates to return toward normal is observed during the 30 days before the second amputation, but this is relatively small. After the second amputation there is, however, a sharp decrease in the number of abnormal limbs. The unirradiated limbs tend to develop a small proportion of abnormalities, and if this is corrected for, as in figure 2, there appears to be almost complete recovery after the second amputation.

The probability that the decrease in abnormalities of the regenerates after 30 days was a result of the second amputation prompted another experiment (Series RL) in which half the limbs underwent a second amputation at 30 days, the other half being allowed to recover undisturbed, if they would. Ninety-two larvae of *A. opacum,* average body length 27.0 mm (S.D. = 2.1) were used in this experiment. The results, presented in figure 3, show clearly that the second amputation increased the return of the limb toward normal, the proportion of abnormalities remaining nearly constant in the limbs which were amputated only once. In this experiment the unirradiated limbs showed an increasing number of abnormalities, possibly associated with the approach of metamorphosis. Again, correction, as in figure 4, indicated a high degree of return toward normal of the irradiated limbs after the second amputation.

There appear to be certain quantitative discrepancies between the results of these two experiments which need to be considered. The recovery from abnormality after the second amputation is less marked in Series RL than in Series LA. In both these experiments there is some return toward normal in the first 30 days after amputation, whereas in the experiment carried out earlier with *A. punctatum* (Blum et al., '57) there was no apparent recovery before the time at which the observations ended. The latter difference may be accounted for, in part, in terms of difference in observational criteria, since the observations were made by different persons. Species differences might also be invoked to explain some of the variation.

It is to be pointed out, however, that the amount of tissue affected by the ultraviolet radiation may be a very important



Figures 1 to 4

factor in such experiments, since the radiation is strongly absorbed by the tissues, and hence the surface layers receive much more than the deeper layers. The epidermal cells closest to the source swell during the period of irradiation, and, the surface area not being enough to accommodate them in their enlarged state, they separate and are carried away. Presumably these cells are killed outright by the radiation; at any rate, they are effectively removed from participation in regeneration. The epidermal cells on the surface of the limb away from the source of radiation remain histologically unchanged, and there are no marked immediate changes in the subepidermal tissues. In the experiments of Rieck ('54) on irradiated limb buds of *Amblystoma* embryos, the subepidermal tissues showed degenerative changes, which were greatly decreased by photorecovery, indicating that the ultraviolet radiation has effect at some depth. But, there is no direct means of determining quantitatively the amount of the tissue of the limb that is affected, nor the degree of effect in different types of cells. It is clear, however, that mere size of the limb may be an important factor in determining the magnitude of the changes undergone by the limb after irradiation — even the qualitative character of the observed response, since this may depend upon the proportion of the different tissues involved.

The difference in dimensions of the limb varies with the age of the animal, and probably with other factors, such as nutrition and temperature during development; and such variation may be more important than species difference *per se.* in determining the quantitative results. It seems likely that the character of some of the abnormalities we have observed, e.g., the development of supernumeraries in the elbow and shoulder regions, result largely from lack of uniformity in the effects on different tissues, there being always a certain proportion of normal or only slightly affected cells that remain after the irradiation. In this case, photorecovery may be thought of as acting to increase the proportion of normal cells and thus to decrease the proportion of abnormalities (Blum et al., '57) and of supernumeraries (Butler and Blum, '55). Differences in the rate and extent of regression of irradiated limbs receiving the same incident dosage have been observed, which are also explainable on the basis of difference in size of limb and hence in effective dose (Butler et al., '57).

Throughout our experiments, every effort has been made to have the animals as uniform as possible for a given experiment. But, for a variety of reasons, this uniformity could not be maintained from experimnet to experiment. There has always been a satisfying degree of consistency in the general character of the results, but there have been various quantitative differences such as the ones cited above, which probably have resulted from the use of somewhat dissimilar animal material. It seems highly possible that this nonuniformity between experiments has been largely due to differences in the size of the parts irradiated.

With the tendency toward recovery after amputation, it was difficult to determine whether a given pattern of abnormality was carried over in the regenerate, but this has not appeared to be the case. In the following studies on supernumeraries it will be seen that the exact pattern is not, as a rule, duplicated by the regenerate after amputation.

Regeneration of supernumeraries. A total of 79 supernumeraries which developed after localized irradiation of the elbow were amputated: 60 were on limbs of *A. punctatum* (series EA); 19 in *A. opacum* (series ER). In only one case did the amputated supernumerary fail to regenerate. Of the 78 cases which regenerated, 12 of the *A. punctatum* series have not been taken into consideration in our statistical analysis, for the reasons that either multiple amputations were made, or the case history was incomplete.

The patterns of supernumerary growths at the elbow ranged from the presence of a single digit, through a variety of types of hands possessing 2, 3, or 4 digits, to one case which had a hand with a total of 6 digits. Figure 5 illustrates three individual cases (ER 100; ER 61; ER 218). The upper row of drawings (A_1, B_1, C_1) shows the fully-formed supernumeraries, and the lower row (A_2, B_2, C_2) the regenerates (R) which were formed after amputation (at the level of the double-pointed arrow in each case). In none of these cases was the original pattern of the supernumerary duplicated. In one instance a three-digit supernumerary (A_1) gave rise to a spike-like digit (A_2); in another, a similar three-digit supernumerary (B_1) formed a two-digit regenerate (B_2); and, in another a 5-digit supernumerary (C_1) produced a two-digit regenerate (C_2). Although there were many cases, as pointed out below, in which a single spike-like supernumerary, after amputation, regenerated another similar structure, seldom if ever, was the precise configuration and proportions of the original supernumerary duplicated in the regenerate.

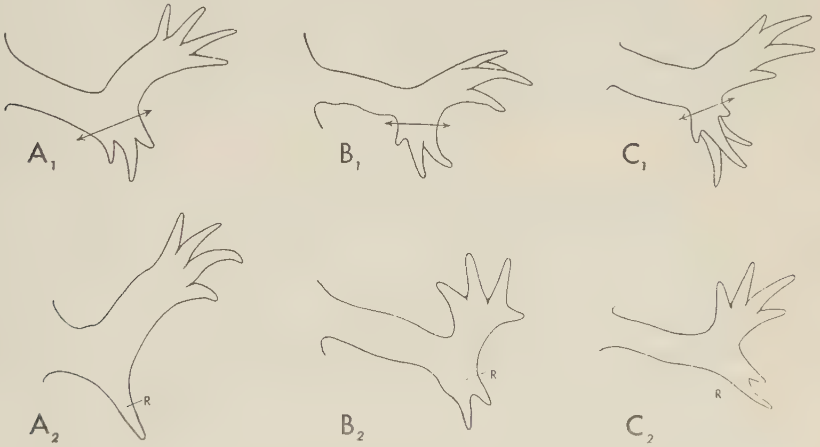


Fig. 5 Drawings A_1, B_1, C_1 show three limbs possessing supernumerary growths; double-pointed arrows indicate levels of amputation. Drawings A_2, B_2, C_2 show the regenerates (R) which developed after the amputations.

Table 1 shows the distribution of the number of digits in the supernumerary limbs before and after amputation. It is to be noted that a small number of digits is characteristic of both the original supernumeraries and the regenerates; in both cases a large majority of the appendages are single spike-like digits.

If there were perfect matching of digits between the original supernumerary and the regenerate after amputation, all numbers would fall along the diagonal from top left to bottom right. This is obviously not the case; however, the distribution in the regenerates seems to bear some relationship to that in the original supernumeraries. For purposes of statistical analysis, table 1 has been condensed to give table 2, where the number of digits before and after amputation has been split into two categories; one digit, and two or more digits. A standard χ^2 analysis of table 2, based on the assumption of independence of digital number before and after amputation, yields (after making Yates' continuity correction) a χ^2 value of 9.15, which is significant at the one percent level.[3] Thus, one rejects the hypothesis that the number of digits in the regenerate is independent of the number of

TABLE 1

Experiments EA and ER

DIGITS AFTER AMPUTATION	DIGITS BEFORE AMPUTATION					
	1	2	3	4	5	Total
1	30	7	4	0	0	41
2	7	7	3	0	1	18
3	1	1	1	4	0	7
4	0	0	0	0	0	0
5	0	0	0	0	0	0
Total	38	15	8	4	1	66

TABLE 2

Experiments EA and ER

DIGITS AFTER AMPUTATION	DIGITS BEFORE AMPUTATION		
	1	$\geqq 2$	Total
1	30	11	41
$\geqq 2$	8	17	25
Total	38	28	66

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[3] We are indebted to Dr. Roger Pinkham for the statistical analysis of these data.

digits in the supernumerary before amputation. That is, there is some tendency for the regenerate to have the same number of digits as the original supernumerary.

The explanation of this tendency is not altogether clear. Why, if the regenerate forms according to the digital pattern laid down in the original supernumerary, is there not much more complete matching than is observed? The data seem to suggest that the regenerate from a supernumerary with a large number of digits is likely to have a larger number of digits than the regenerate from a supernumerary with a small number of digits. It may be suggested that this reflects the size of the original supernumerary, particularly the area of the amputation surface, rather than the actual digital pattern.

SUMMARY

Forearms of larval *Amblystoma* were irradiated with ultraviolet light, then amputated through the distal region of the radius and ulna three days later. A high proportion of abnormal regenerates resulted. A second amputation, 30 days after the first, resulted in a high proportion of recovery toward normal.

Localized irradiation of the elbow produced a high percent of supernumerary growths possessing varying numbers and configurations of digits, single digits predominating. After amputation of these supernumeraries regeneration occurred. The pattern of the regenerates did not duplicate the original pattern of the supernumerary, but a tendency to produce the same number of digits was evident.

In all cases animals were kept in the dark for three days after irradiation; thus photorecovery was not a factor.

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SUPPLEMENT

SOCIETY OF GENERAL PHYSIOLOGISTS

Abstracts of papers presented at the thirteenth Annual Meeting,[1] Marine Biological Laboratory, Woods Hole, Massachusetts, June 9, 10, and 11, 1958.

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[1] Abstracts or summaries of programs of previous meetings will be found in Biol. Bull., *91:* 236; *93:* 222; *95:* 281; *97:* 267; *99:* 308 (1946–50); Science, *114:* 699 (1951) and *118:* 768 (1953); J. Nat. Cancer Inst., *13:* 1399 (1953); and J. Cell. and Comp. Physiol., *44:* 327; *46:* 353; *48:* 329; *50:* 349–356; (1954–57).

ABSTRACTS

The Effect of Metabolic Poisons on the Pigments of *Euglena Gracilis*.

Charles Greenblatt and Norman Sharpless, National Institutes of Health, Bethesda, Maryland.

Darkness, lack of oxygen, and an acidic medium promote formation of a pheophytin-like pigment from chlorophyll in *Euglena*. In an attempt to elucidate the metabolic basis for this alteration, we have employed a number of metabolic poisons. Dinitrophenol, in an acidic medium, produces similar but more drastic pigment changes. The extent of pigment decay is dependent on dinitrophenol concentration, pH, light, and oxygen. Inhibition of the contractile vacuole, volume changes of the organism, and major structural alterations of the chloroplast accompany the pigment decay.

Of other metabolic poisons investigated, pentachlorophenol, iodoacetamide, sodium fluoride, and sodium azide produce similar pigment degradation. Malonic acid is ineffective.

This general type of pigment decay is most simply explained as an accumulation of acid in the organism. The active transport of hydrogen ions in relation to the contractile vacuole and cell wall will be considered.

Temperature and the Non-luminescent Oxidation of Cypridina Luciferin.[1]

Aurin M. Chase, Fred S. Hurst and Howard J. Zeft, Princeton University, Princeton, N. J.

Chase and Lorenz (J. Cell. and Comp. Physiol., *25:* 53, 1945) described the effect of temperature on the luminescent reaction of *Cypridina* luciferin and luciferase by an equation representing two concurrent first order processes. One was the luciferase-catalyzed luminescent reaction, and the other a spontaneous non-luminescent oxidation of luciferin. A recent study of the non-luminescent oxidation, by itself, at different temperatures shows that this equation probably does not represent the complete mechanism even though it fits the data. The present experiments confirm that the non-luminescent oxidation of luciferin is, indeed, markedly affected by temperature, but not enough to account completely for the results of Chase and Lorenz. There are evidently at least three factors involved in the decrease in total light observed with increased temperature. Two of these are the inactivation of luciferase and the non-luminescent oxidation of luciferin. In addition increased temperature may reduce the total light obtainable by exerting a quenching effect upon the emitting molecule or complex, just as certain ions are known to do. The effects of temperature on the activity of luciferase and on the non-luminescent oxidation of luciferin cannot, consequently, be separately calculated from the equation of Chase and Lorenz, unless it be assumed that the latter reaction is itself greatly accelerated by luciferase or by some catalyst associated with it. It has not as yet proved possible to test such an assumption experimentally.

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[1] Supported in part by a National Science Foundation grant.

FURTHER EVIDENCE CONCERNING THE CATCH MECHANISM IN MOLLUSCAN MUSCLES.

William H. Johnson, University of Illinois, Urbana, Illinois.

The time course of relaxation following the contractile response of the anterior byssus retractor muscle of the sea mussel, *Mytilus edulis* is dependent on the type of stimulus used to elicit the response, as has been shown by Winton and others. The muscle relaxes slowly following catodal direct current (DC) stimuli, while pulsed or alternating currents (AC) elicit rapid relaxation. Essentially two hypotheses have been advanced to explain the delayed relaxation following DC stimuli. One postulates continued activity within an intrinsic neuro-muscular system which maintains contraction following DC stimuli by a tetanic activation of the contractile system by which is not active following AC stimuli. The second postulates a slowly reversible change of the mechanical properties of the contractile system capable of maintaining tension or shortening and which is activated by DC stimuli. Using a triangular electrode of the type originally described by Taylor, which can produce uniform currents in either direction through the membrane over 85–90% of the length of the muscle, we have been unable to detect electrical activity at high amplifier gains during the slow relaxation produced when cathodal membrane currents are used to activate the muscle. Quick release of the muscle at any time during the period of slow relaxation gave no indication of the presence of an ''active state''; this state could clearly be detected during the period of stimulation. Cathodal DC stimulation at muscle lengths less than rest length results in little contractile activity, but the stiffness of the muscle increases fully as much as it does when such stimuli are delivered at rest length. The contractile tension at rest length is very large, indicating that a change has occurred in the mechanical properties of the muscle which does not parallel the ability to develop tension. The latter results suggest a change within the contractile system itself as the basis for slow relaxation, while the former results seem incompatable with the first of the above mentioned hypotheses.

INDUCED VOLUME CHANGES IN CELL NUCLEI.

Clifford V. Harding and Carl Feldherr, University of Pennsylvania, Philadelphia 4, Pa.

Volume changes in cell nuclei were induced by microinjection of various solutions into the cytoplasm of immature oocytes of *Rana pipiens*. A solution of 9 parts isosmotic KCl and one part isosmotic NaCl, as well as a solution 1.2 times this concentration, caused a significant increase in nuclear volume. Injection of 2% and 4% polyvinylpyrrolidone (PVP), of molecular weight 40,000, as well as 4% bovine serum albumin (BSA), molecular weight 65,360, caused a significant decrease in nuclear volume. Concentrations of 1–1.5% PVP and 2% BSA caused no significant change in nuclear volume when injected into the cytoplasm. Injection of 0.5% PVP caused an increase in nuclear volume. All these solutions, which contained KCl and NaCl, were isosmotic with amphibian Ringer, and had a pH of approximately 6.5. The effects of PVP and BSA on nuclear volume are interpreted as osmotic effects, and the concentrations which cause no significant change in nuclear volume are thus considered to be isotonic. The nuclear membrane is visualized as having some of the properties of an ultrafilter, allowing the free penetration of relatively small molecules, but not the larger molecules. This concept of the nuclear membrane would account for the long-known properties of cell nuclei as osmometers. Further analysis of the osmotic behavior of nuclei may also be of value in the design of more ''physiological'' media for use in studies on isolated nuclei and possibly in studies on nuclear transplantation.

CATION TRANSPORT IN HIGH AND LOW POTASSIUM SHEEP RED CELLS.

D. C. Tosteson and J. F. Hoffman, National Institutes of Health, Bethesda, Maryland.

Some individual sheep have red cells with high potassium and low sodium concentrations (HK type) whole other sheep have red cells with high sodium and low potassium concentrations (LK type). Recent evidence of Evans suggests that the LK character is inherited as a Mendelian dominant. A detailed comparison of the potassium and sodium transport processes in HK and LK sheep red cells will be described. Measurements designed to separate active transport, exchange diffusion and passive diffusion components of K and Na fluxes by techniques involving variations in the composition of the medium, and the use of different substrates and strophanthidin permit the following conclusions: (1) An active transport component of K influx occurs in LK as well as HK cells, but is 4 times greater in the latter type. (2) An active transport component of Na outflux has been identified in HK but not in LK cells. (3) A large fraction of the total Na flux occurs by exchange diffusion in both HK and LK cells. (4) The passive permeability to K and Na of HK and LK cells differ markedly. LK cells have a greater passive permeability to K and a smaller passive permeability to Na than do HK cells. Thus it appears that a single gene controls both the magnitude of active transport and the resistance to passive diffusion of Na and K in sheep red cells. These and other results will be discussed in relation to the regulation of cell salt and water content and to the coupling of active transport processes to metabolism.

OBSERVATIONS ON THE MECHANICAL BEHAVIOR OF WET AND DRY RAT TAIL TENDON.

Bernard Rigby, Nishio Hirai, John D. Spikes and Henry Eyring, University of Utah, Salt Lake City, Utah.

The mechanical behavior of rat tail tendon in saline is reproducible at room temperature as long as the strain does not exceed 1%. The properties are unaffected by temperatures up to approximately 38°C. Just above this temperature, however, significant changes are observed. The behavior of dry tendon (in paraffin oil) has been compared with that of tendon in saline, and x-ray studies have been made of both wet and dry tendon as a function of temperature and strain.

FURTHER REPORT ON SOME MOLECULAR ASPECTS OF TUMOR-HOST RELATIONSHIPS.

Vernon Riley, Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research and Memorial Center for Cancer and Allied Diseases, New York, N. Y.

Transplantable melanomas of mice and of hamsters have been used as neoplastic models to test the anti-tumor properties of compounds that can react with a specific tumor product. An oxygen consuming reaction takes place between such a product, dihydroxyphenylalanine (DOPA), and para, meta, or ortho phenylenediamine (PPDA, MPDA, OPDA). The *in vitro* reaction can be demonstrated manometrically with the pure compounds, or by adding PPDA to melanoma extract or to the plasma or urine of advanced pigmented melanoma patients. Indirect evidence of combination of the phenylenediamines with melanoma components can be demonstrated by comparison of the survival times of tumor-bearing and normal animals after they have been challenged with a single lethal dose of PPDA, MPDA, or OPDA. Differential chronic toxicity effects associated with the presence or absence of melanoma in OPDA treated animals have also been observed. When these compounds were administered in chemotherapeutic experiments, significant inhibition was obtained against the Cloudman S91 melanoma, the Harding-Passey melanoma, the Ehrlich solid carcinoma, and the recently reported metastasizing melanomas of hamsters. Degree of tumor inhibition differed with change in relative position of the NH_2 groups with the melanostatic effects increasing in the following isomeric order: para, meta, ortho. The toxicity of the compounds decreased in the same order, thus permitting the administration of correspondingly higher doses, which may account for their differential effectiveness. A maximum of approximately 90% inhibition of S91 melanoma growth was obtained when the hosts were injected intraperitoneally with ortho phenylenediamine (free base) five days per week at doses of 200 to 250 mg per kg per day. The possible interrelationships of the *in vitro* reactions with the various *in vivo* phenomena are under study.

MITOCHONDRIAL ENZYME ACTIVITY IN LIVER AND BRAIN TISSUE OF HYPOTHYROID AND HYPERTHYROID RATS.[1]

Samuel R. Tipton, Georgia St. Amand and Marilyn Zirk, The University of Tennessee, Knoxville, Tennessee.

The activities of succinic dehydrogenase (SDH-ase), glutamic dehydrogenase (GDH-ase), and cytochrome oxidase per mg mitochondrial nitrogen and mg mitochondrial dry weight were increased in mitochondria separated from livers of hyperthyroid rats above the activities in mitochondria from thyroid-ectomized animals. The increase in activity of these enzymes after the animals were maintained for 10–12 days on a diet of 1% thyroid powder, or subcutaneous injections of doses of 0.3 mg thyroxine every other day (1½–3 mg total dose) varied from 7 to 36%. There was a small but significant increase in nitrogen, but the total mitochondrial count was not significantly different in the two thyroid states. The enzyme activity per million mitochondria is higher in the fraction from the hyperthyroid animals but the difference is not significant. It appears that the hormone affects specific enzyme activity and may have little effect on the number of mitochondria. Data are also presented on the swelling of brain mitochondria when treated with adequate concentrations of thyroxine *in vitro.* Since brain tissue is ''refractory'' to the hormone in vivo, the suggestion is made that the thyroxine effect on mitochondrial volume is a non-specific one.

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